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GRANT NUMBER DAMD17-96-1-6019

TITLE: Molecular Analysis of Bcl-xS-Induced Apoptosis in Breast Cancer

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REPORT DATE: July 1999

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

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DTIC QUALITY INSPECTED 4

20000111 031

REPORT DOCUMENTATION PAGE

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OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

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|---|---|--|---|--|
| 1. AGENCY USE ONLY (Leave blank) | | 2. REPORT DATE July 1999 | 3. REPORT TYPE AND DATES COVERED Annual (1 Jul 98 - 30 Jun 99) | |
| 4. TITLE AND SUBTITLE Molecular Analysis of Bcl-xS-Induced Apoptosis in Breast Cancer | | | 5. FUNDING NUMBERS DAMD17-96-1-6019 | |
| 6. AUTHOR(S) Gabriel Nunez, M.D. | | | | |
| 7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Michigan Ann Arbor, Michigan 48103-1274 | | | 8. PERFORMING ORGANIZATION REPORT NUMBER | |
| 9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Frederick, Maryland 21702-5012 | | | 10. SPONSORING/MONITORING AGENCY REPORT NUMBER | |
| 11. SUPPLEMENTARY NOTES | | | | |
| 12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited | | | 12b. DISTRIBUTION CODE | |
| 13. ABSTRACT (Maximum 200) A major genetic event that occurs in the pathogenesis of breast carcinoma involves alterations in the Bcl-2 survival pathway. In the original proposal, we proposed studies to determine the mechanism involved in Bcl-xS-mediated apoptosis, to characterize cellular proteins that interact with Bcl-xS using biochemical and genetic approaches, and to use a transgenic model of Bcl-xS expression in the breast to assess the requirement for Bcl-2/Bcl-XL in the maintenance of normal breast epithelia and tumor growth. During the last year, we have further studied the apoptotic pathway induced by Bcl-xS. The analysis showed that Bcl-xS kills cells, at least in part, through an Apaf-1-caspase-9 pathway. Bcl-xS associates with Bcl-xL via its BH3 domain and with Apaf-1 possibly through Bcl-xL. We have cloned and characterized a novel Apaf-1 cDNA form that, in contrast to the originally described Apaf-1, activates procaspase-9 in a cytochrome c and dATP-dependent manner. Finally, transgenic mice expressing Bcl-XS in the breast have been crossed with oncogenic erb2-B transgenic mice to assess the effects of Bcl-xS on breast cancer development. | | | | |
| 14. SUBJECT TERMS Breast Cancer | | | 15. NUMBER OF PAGES 40 | |
| | | | 16. PRICE CODE | |
| 17. SECURITY CLASSIFICATION OF REPORT Unclassified | 18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified | 19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified | 20. LIMITATION OF ABSTRACT Unlimited | |

FOREWORD

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INTRODUCTION

Cancer is the result of multiple genetic events, including activation of oncogenes and inactivation of tumor suppressor genes. The protein products of the former are often mitogens, whereas the products of the latter suppress proliferation. It is becoming increasingly apparent that tumor suppressor genes like p53 function in part by activating an apoptotic death pathway. In addition, certain oncogenes such as *bcl-2* appear to contribute to tumor development primarily by promoting abnormal cell survival via an apoptosis inhibitory signal. Thus, disruption of the apoptosis pathway appears integral to many malignancies including breast cancer. Furthermore, treatment of cancer with chemotherapy or radiation therapy is limited by the emergence of tumor cells resistant to these therapies. This resistance limits our ability to successfully treat these neoplasms.

bcl-2, the first member of an evolutionarily conserved family of apoptosis regulatory genes, was initially isolated from the t(14; 18) chromosomal translocation found in human B-cell follicular lymphomas, and was subsequently shown to repress cell death triggered by a diverse array of stimuli (1-2). Several members of the family, including Bcl-2, Bcl-xL, Bcl-w, Mcl-1, and A1/Bfl-1, share conserved regions termed Bcl-2 homology domain 1, 2, 3, and 4 (BH1, BH2, BH3, and BH4), and function by repressing apoptosis (1-4). In contrast, structurally related proteins, including Bax, Bak, Bad, Bik/Nbk, Bid, Hrk, Bim, and Bok/Mtd, activate apoptosis (5-11). Biochemical and functional analyses have revealed that these pro-apoptotic proteins require the conserved BH3 region to interact with Bcl-2/ Bcl-xL, and activate apoptosis in transient assays (5-11). Moreover, NMR studies have demonstrated that the BH3 domain of Bak interacts with a hydrophobic cleft formed by the conserved BH3 and BH1 regions of Bcl-xL (12). To date, all death-promoting Bcl-2-related proteins heterodimerize with Bcl-2, Bcl-xL, or Mcl-1, suggesting that these molecules promote cell death at least in part by interacting with and antagonizing Bcl-2, Bcl-xL, and Mcl-1 (5-11). The biochemical process by which pro-survival Bcl-2 family members regulate cell death is poorly understood. It has been proposed that pro-survival family members regulate apoptosis by maintaining the integrity of the mitochondria (13). In addition, these Bcl-2 family proteins have been proposed to regulate apoptosis via physical interactions with central caspases through adaptor molecules, such as Apaf-1 and CED-4 (14-15).

A major genetic event that occurs in the genesis and/or progression of breast carcinoma involves alterations in the pathway of apoptosis. In breast cancer, one of the most common abnormalities is deregulated expression of Bcl-2 or Bcl-xL proteins. Up to 90% of cancers originating from breast overexpress Bcl-2 or Bcl-xL (16-17). We hypothesize deregulated expression of these proteins plays a critical role in the maintenance of breast cancer cells and resistance of tumor cells to therapy-induced apoptosis. To determine the role of the Bcl-2 family of proteins in maintaining cancer cell viability, we constructed a recombinant adenovirus vector that expresses Bcl-xS, a dominant inhibitor of Bcl-2 and Bcl-xL. Even in the absence of an exogenous apoptotic signal, this recombinant virus specifically and efficiently activates apoptosis in human carcinoma cells arising from multiple organs including breast, colon, stomach and sympathetic nervous tissue (18). Based on these results, we hypothesize that apoptotic signals are constitutively expressed

in proliferating cancer cells and perhaps in normal cells, although repressed by members of the Bcl-2 family of proteins. In this proposal, we proposed studies (i) to determine the mechanism involved in Bcl-xS-mediated apoptosis using the *bcl-xS* adenovirus to dissect molecular interactions of the Bcl-2 regulatory pathway; and (ii) to characterize cellular proteins that interact with Bcl-xS using biochemical and genetic approaches. The studies outlined in this proposal may provide novel insight into the apoptosis pathway and lead to alternative therapeutic strategies for the treatment of breast cancer and other malignancies.

BODY OF THE ANNUAL REPORT

Technical Objective #1: Further characterization of the interaction of Bcl-xS with Bcl-2, Bcl-xL and Bax in breast cancer cells.

Task 1.1: Determine whether Bcl-xS associates with Bcl-xL and/or Bcl-2 *in vivo*. This task has been completed.

Task 1.2: Determine whether Bcl-xS disrupts the interaction of Bcl-xL with Bax and Bak. This task has been completed.

Task 1.3: Determine whether Bcl-xS functions by association with other proteins that regulate apoptosis

Recent experiments with nematode CED-9, CED-4 and CED-3 have suggested that Bcl-2 and Bcl-xL regulate apoptosis by interacting with and inhibiting caspases (death proteases) through Apaf-1, a mammalian homologue of the *C. elegans* CED-4 protein (19-21). These observations suggested that Bcl-xS might antagonize Bcl-xL by interfering with the ability of Bcl-xL to inactivate the caspase regulator Apaf-1. Consistent with this, we and others have shown that Bcl-xL associates with Apaf-1 (14-15). Apaf-1 is a key component of a major pathway of apoptosis that is activated by intracellular cell death signals that induce mitochondrial damage and cytochrome c (22). In the presence of both cytochrome c and dATP, Apaf-1 undergoes a conformational change such that it binds and activates procaspase-9 (23). Activated procaspase-9 activates effector caspases such as caspase-3, an event that mediates the execution of cell death (23).

Bcl-xS killing is repressed by caspase inhibitors and dominant negative caspase-9

To assess whether Bcl-xS can promote apoptosis via the Apaf-1/caspase-9 pathway, we expressed Bcl-xS in the presence or absence of caspases inhibitors including baculovirus p35 and cell-permeable modified peptide BOC-fmk and more importantly with a dominant interfering mutant of caspase-9. In figure 1, it is shown that normal mouse embryo fibroblasts (MEFs), Bcl-xS killing is inhibited by Bcl-2 and Bcl-xL (as previously shown), but also by p35 and BOC-fmk, and more importantly by dominant

negative caspase-9. Given our previous observation that Bcl-xS and Bcl-xL can bind Apaf-1, this data would be consistent with a model in which Bcl-xS, killing acts, at least in part, through the Apaf-1/caspase-9 pathway of apoptosis.

Bcl-xS killing requires Apaf-1 and caspase-9 in MEFs

To further verify these results, we used MEFs derived from mutant mice in which the Apaf-1 or caspase-9 genes have been ablated by homologous recombination (24-45). Bcl-xS killed wild-type (WT) MEFs, as detected by a great reduction of cells that expressed a reporter LacZ gene (blue cells), but not Apaf-1 ^{-/-} or caspase-9 ^{-/-} deficient MEFs (Figure 2). These results further indicate that Bcl-xS can kill cells via an Apaf-1/caspase-9 pathway of apoptosis.

The BH3 domain of Bcl-xS is required for binding to Bcl-xL

We showed in the last report that Bcl-xS associated with Apaf-1 but it was unclear if the binding was indirect and mediated by endogenous Bcl-xL or another protein present in the cell. To assess the former, we determined whether the binding of Bcl-xS to Apaf-1 was enhanced by Bcl-xL expression. In these experiments, HA-tagged Bcl-xS and Myc-tagged Apaf-1 mutant constructs were transfected into 293T cancer cells in the presence or absence of Flag-tagged Bcl-xL. Immunoprecipitation of Bcl-xS complexes with anti-HA antibody revealed that the binding of Bcl-xS to Apaf-1 was enhanced by Bcl-xL, as determined by immunoblotting for Apaf-1 with anti-Myc antibody (Figure 3). This results cannot be explained by increased expression of Bcl-xS protein as assessed by immunoblotting with anti-HA antibody (Figure 3, middle panel). We showed in the last report that a Bcl-xS mutant with deletion in the BH3 domain failed to kill cells. We have followed up these observations to assess whether Bcl-xS requires its BH3 domain for binding to Bcl-xL. In these experiments, Flag-tagged Bcl-xL and various HA-tagged Bcl-xS mutant constructs were transfected into 293T cancer cells and Bcl-xL complexes were immunoprecipitated with anti-Flag antibody. In Figure 4, we showed that the BH3 domain of Bcl-xS (residues 116-132) is required for binding to Bcl-xL. Bcl-xS (with deletion of residues 116-132) was expressed as detected by immunoblotting of total NP-40 lysates (panel B of Figure 4). Furthermore, these results were specific in that other Bcl-xS mutants in which other regions of the molecule were deleted still bound to Bcl-xL (Figure 4A).

Identification of Apaf-1 Splice Variants

The studies with Bcl-XL has been performed with Apaf-1S (see Figure 5A). However, we were unable to demonstrate cytochrome c binding (see below) to the Apaf-1S isoform originally described by Zou *et al.* (22) and also cloned by us from HeLa cDNA (Figure 5). We also noticed that endogenous 293T Apaf-1 protein appeared to migrate somewhat slower than transfected Apaf-1S (see below in Figure 5E), and in our hands we were unable to demonstrate cytochrome c/dATP-dependent *in vitro* activation of procaspase-9 by the Apaf-1S isoform (see below in Figure 2). We, therefore, used RT-PCR to clone other potential full length Apaf-1 cDNAs from 293T cells. Two full length Apaf-1 cDNAs were cloned from 293T cells and were identical to the Apaf-1S isoform, except that they contained an 11 amino acid insert (GKDSVSGITSY) at position 98 between the CARD and ATPase domain and a 43 amino acid WD-40 repeat (WDR) inserted between

the fifth and sixth existing WDRs of Apaf-1S (Figure 5). The presence of the N-terminal insert is consistent with the utilization of an alternative exon donor site in exon 3 and a single acceptor site in exon 4 (Genbank accession numbers AF098871 and AF098873, respectively). The presence of the additional C-terminal WDR is consistent with the utilization of an additional exon 17a (Genbank accession numbers AF117658 and AF117659). Recently, Zou *et al.* (26) have also reported the cloning of this Apaf-1 cDNA from HeLa cells. For consistency and clarity in this paper, we have termed this isoform Apaf-1XL. This was done to distinguish it from two other alternative human Apaf-1 cDNA splice variants (Figs. 5B,C, D) that are also longer than the originally identified Apaf-1S (Figure 5A). We constructed these two alternative Apaf-1 cDNAs using the Apaf-1S and Apaf-1XL cDNAs as described in Experimental Procedures. For clarity in this paper, we have termed them Apaf-1LC (Long C-terminus: containing the additional WDR, but lacking the N-terminal insert) and Apaf-1LN (Long N-terminus: containing the N-terminal insert, but lacking the additional WDR) (Figure 5A).

Expression Apaf-1 Isoforms in Tissues and Cell Lines

All of the human Apaf-1 cDNAs described have been isolated from tumor cell lines. To determine if multiple Apaf-1 cDNAs are present in normal human tissues, we performed full length Apaf-1 PCR analysis on cDNAs generated from normal human tissue RNAs (Figure 5B). This analysis demonstrated the existence of at least two Apaf-1 cDNA forms. The larger form co-migrated with the cloned Apaf-1XL fragment, while the smaller form migrated slightly above that of Apaf-1S (Figure 5B). Restriction mapping of each of these gel purified full length Apaf-1 PCR products confirmed their identities as Apaf-1 cDNAs. Because of limited gel resolution, minor amounts of other Apaf-1 cDNAs may have been present but not detectable. To better examine the relative amounts of the different Apaf-1 forms, we performed PCR analysis of the human tissue cDNAs using two sets of primers that flank the two different insertions. Primers N1 and N2 flank the N-terminal 11 amino acid insertion, while primers C1 and C2 flank the additional WDR (Figure 5A). PCR analysis using primers N1 and N2 showed that in all tissues the great majority of the products (> 80%) contained the 11 amino acid N-terminal insertion, as determined by comparison with control reactions containing various ratios of Apaf-1XL and Apaf-1S DNAs (Figure 5C). PCR analysis using primers C1 and C2 showed that in all tissues both types of products are represented, although the relative amounts of the two types varied among the tissues (Figure 5D). A compilation of the PCR results from Figures 1C and 1D suggests that the major full length Apaf-1 cDNAs observed in most of these tissues appears to be Apaf-1XL (containing both N-terminal and C-terminal insertions). At the level of mRNA expression, tissues such as bone marrow, colon and spleen appear to have roughly equal amounts of Apaf-1XL and Apaf-1LN (containing just the N-terminal insertion), while tissues such as brain, kidney, stomach, and skeletal muscle express more Apaf-1XL.

To determine whether multiple Apaf-1 isoforms are also expressed at the protein level, we examined multiple cell lines by immunoblotting using a polyclonal anti-Apaf-1 antibody. Lysates of 293T cells transiently transfected with the different Apaf-1 forms (described in Figure 5A) were run as controls. The major immunoreactive band in each of the cell lines co-migrated with the Apaf-1XL form (Figure 5E), which is consistent

with the data from the mRNA analysis of human tissues identifying Apaf-1XL as the major form expressed (Figures 5B, C, D). As in the tissue mRNAs, multiple Apaf-1 protein isoforms were also expressed in these cell lines (Figure 5E). These other bands appear to co-migrate with the Apaf-1LN and Apaf-1S forms, however, exact identification would require either protein sequencing or isoform-specific antibodies. Similar results were obtained when lysates from normal breast tissue and two breast cancer cell lines were immunoblotted with anti-Apaf-1 antibody (data not shown). Immunoblotting of these lysates with another polyclonal anti-Apaf-1 antibody (Cayman Chemical Company) confirmed these results.

Cytochrome c/dATP-dependent *in vitro* activation of procaspase-9 requires the additional WD-40 repeat

Purified Apaf-1 has been reported to activate procaspase-9 in a cytochrome c and dATP-dependent fashion (23). To determine if the newly identified Apaf-1 cDNAs also share this activity, and to determine the role of the N-terminal and C-terminal insertions, the four full length Myc-tagged Apaf-1 constructs (Figure 5) and the originally described untagged Apaf-1S were expressed in 293T cells. Cytosolic extracts of these cells were prepared and anti-Apaf-1 immunoblotting confirmed comparable expression of each of the transfected Apaf-1 forms (Figure 6). Ten μ g of the fresh cytosolic extracts were also analyzed for their ability to activate procaspase-9 *in vitro*. Extracts were diluted so that endogenous Apaf-1 activity could not be detected. As shown in Fig. 6, only Apaf-1XL and Apaf-1LC containing the extra WDR, but not those isoforms lacking it, were able to activate procaspase-9 in a cytochrome c and dATP-dependent fashion as indicated by the appearance of the intermediate p35 proteolytic fragments. These same results were obtained using one or thirty μ g of cytosolic extracts (data not shown). Due to the presence of low levels of dATP or ATP in the cytosolic extracts, we confirmed the requirement for ATP by the addition of the non-hydrolyzable ATP analogue, ATP- γ -S, which almost completely inhibited the cytochrome-c/dATP-dependent activation of procaspase-9 (Figure 6). Although the original untagged Apaf-1S was previously reported to activate procaspase-9 in a cytochrome c/dATP-dependent fashion (23), we were unable to detect such activity under our assay conditions (Figure 6).

The additional WD-40 repeat of Apaf-1 is necessary but not sufficient for the binding of cytochrome c

The apparent requirement of the extra WDR for cytochrome c/dATP-dependent activation of procaspase-9 prompted us to examine the ability of the different Apaf-1 forms to bind cytochrome c. As shown in Fig. 3A, following anti-Myc antibody immunoprecipitation in the presence or absence of cytochrome c, dATP or ATP- γ -S and immunoblotting with mouse anti-cyt c antibody, only the Apaf-1 constructs Apaf-1XL and Apaf-1LC, containing the extra WDR, bound to cytochrome c. This cytochrome c binding also required ATP/dATP, as binding was greatly inhibited by the addition of the non-hydrolyzable ATP analogue, ATP- γ -S (Figure 7A). The N-terminal 11 amino acid insert was not required for cytochrome c binding, as the form Apaf-1LC, lacking the N-terminal insert, bound cytochrome c (Fig. 3A). Cytochrome c binding to the C-terminal deletion mutant, C+ (Apaf-1XL 479-1248), containing the extra WDR was not detected,

suggesting that this region, although necessary (Figure 7A), is not sufficient to mediate cytochrome c binding (Figure 7B). Cytochrome c also failed to bind to either the N+ (Apaf-1XL 1-570) or N- (Apaf-1S 1-559) deletion mutants (Figure 7B). This data is consistent with a model in which the C-terminal WDR region with the additional WDR contains a binding site for cytochrome c that may be unmasked only following a conformational change driven by ATP hydrolysis in the N-terminus.

Cytochrome c/dATP-dependent Apaf-1 Self-association requires the additional WD-40 repeat

We and others have previously shown that Apaf-1 can self-associate to form homo-oligomers (27-29). As our previous studies utilized Apaf-1 protein in NP-40 cellular extracts, we first used NP-40 extracts to compare the four different full length Apaf-1 constructs for their ability to form homo-oligomers. Myc- and HA-tagged isoforms were expressed in 293T cells, lysed in NP-40 buffer, immunoprecipitated with anti-Myc antibody and immunoblotted with anti-HA or anti-Myc antibodies. As shown in Fig. 4A, all four Apaf-1 isoforms were able to self-associate. Anti-HA immunoblotting confirmed similar expression of the HA-Apaf-1 isoforms in the NP-40 extracts. Since these studies were performed in the presence of detergent, we repeated these self-association experiments under more physiological conditions. Cytosolic extracts of Myc- and HA-Apaf-1 isoforms made without detergent were mixed in the presence or absence of cytochrome c, dATP, or ATP- γ -S. Under these conditions, only Apaf-1XL and Apaf-1LC, both containing the extra WDR, underwent cytochrome c/dATP-dependent self-association (Figure 8B). Because Apaf-1LC lacks the N-terminal 11 amino acid insert, this region is clearly not required for cytochrome c/dATP-dependent self-association. Anti-HA immunoblotting confirmed similar expression of the HA-Apaf-1 isoforms. This data is consistent with a model in which cytochrome c binds the Apaf-1 C-terminus with the extra WDR, thus relieving the inhibition of the N-terminus, and allowing Apaf-1 self-association. The requirement of the extra WDR for Apaf-1 self-association is also consistent with our observation that it is required for procaspase-9 activation (Figure 7). Not surprisingly, the two constructs, Apaf-1XL and Apaf-1LC, with the extra WDRs, were capable of forming cytochrome c/dATP-dependent hetero-oligomers (Figure 8B). However, hetero-oligomers between the two major cDNAs detected in human tissues, Apaf-1XL (with the extra WDR) and Apaf-1LN (lacking the extra WDR), were not observed (data not shown).

Cytochrome c/dATP-dependent binding of Apaf-1 to Procaspase-9 requires the extra WD-40 repeat

We and others have previously demonstrated the binding between the originally described Apaf-1S and procaspase-9 (14-15, 23). We, therefore, compared the four full length Apaf-1 isoforms for their ability to associate with procaspase-9 in the presence of NP-40. Each Myc-tagged Apaf-1 construct and HA-procaspase-9 (C287S) were expressed in 293T cells, lysed in NP-40 buffer, immunoprecipitated with anti-Myc antibody and immunoblotted with anti-HA or anti-Myc antibodies. As shown in Fig. 9A, all Apaf-1 isoforms tested bound to procaspase-9. Anti-HA immunoblotting confirmed similar expression of procaspase-9 in each extract. However, when binding was analyzed in cytosolic extracts lacking detergent, in the presence or absence of cytochrome c, dATP

or ATP-g-S, only Apaf-1XL and Apaf-1LC containing the extra WDR bound to procaspase-9 in a cytochrome c and dATP-dependent fashion (Figure 9B). Because Apaf-1LC lacks the N-terminal 11 amino acid insert, this region is clearly not required for cytochrome c/dATP-dependent procaspase-9 binding. The requirement of the extra WDR for procaspase-9 binding is in complete agreement with our observation that this region is also required for procaspase-9 activation (Figure 6). Previous data from our laboratory suggests that the C-terminal WDRs of Apaf-1 can bind and inhibit the ability of the N-terminus to self-associate and to activate procaspase-9 *in vitro* (27). Taken together, the data presented herein suggest a model in which cytochrome c, in the presence of ATP/dATP, binds to the C-termini of only those Apaf-1 isoforms containing the extra WDR, thus relieving the inhibition of the N-terminus, and allowing Apaf-1 self-association, procaspase-9 binding, and the activation of procaspase-9. A biochemical model of caspase-9 activation by Apaf-1 using the newly identified Apaf-1XL has been recently reported by us (30).

As Apaf-1XL and not the originally described Apaf-1 is the functional form, we are currently re-assessing Bcl-XL and Bcl-XS binding to Apaf-1. Also, now that we have an Apaf-1 function to assay (in-vitro caspase-9 activation), we can measure if recombinant Bcl-XS can directly promote Apaf-1/procaspase-9 activation, or whether it functions by preventing Bcl-XL-mediated inhibition of procaspase-9 activation.

Technical Objective #2: Further characterization and purification of p15, a cellular protein that interacts with Bcl-xS.

A mechanism that might explain the apoptosis-promoting activity of Bcl-xS is through binding to an upstream activator or a downstream effector of Bcl-2/Bcl-xL mediated survival. In preliminary results, we provided evidence in the original application that Bcl-xS interacts with a cellular protein, p15, in cancer cells infected with the *bcl-xS* adenovirus. The significance of the Bcl-xS-p15 interaction was unclear. p15 could represent a death effector which is activated by the expression of Bcl-xS. Alternatively, p15 could be a cellular protein required for survival whose activity is inhibited by the Bcl-xS interaction. Clearly, biochemical characterization of p15 and isolation of the p15 cDNA was needed to further assess the role of p15 in Bcl-xS-mediated apoptosis.

Task 2.1: Biochemical Purification and N-Terminal Microsequencing of p15. Initial work was completed. Further work was suspended (see last year report).

Task 2.2: Genetic Screen for Bcl-x-Binding Proteins using the Two-Hybrid Yeast Assay. This task has been completed.

Technical Objective #3: Analysis of Transgenic Mice Expressing Inducible Bcl-XS in the Breast.

Task 3.1: Expression analysis of transgenes

Several transgenic mouse WAP-Bcl-XS lines were identified. Two positive mouse transgenic lines were identified that express the Bcl-XS transgene in the breast (see last year report).

Task 3.2: Histological evaluation of mice

We reported that in both lines, females failed to lactate during the second pregnancy. Histological analysis of breast sections showed that control mice exhibited glandular hyperplasia induced by pregnancy whereas Bcl-xS transgenic females did not (last year report). This finding appear to explain why transgenic females fail to lactate, as their breast tissue did not undergo hyperplasia and differentiation that are required lactation. A possible explanation is that deregulated expression of Bcl-xS mediates apoptosis of epithelial cells that are responsible for glandular hyperplasia associated with the second pregnancy. We performed in-situ TUNEL analysis of breast tissue in control and transgenic mice 10 days after pregnancy. The analysis did not find an increase in apoptosis in transgenic mice over controls (data not shown). However, these results are difficult to interpreter and do not rule out that Bcl-xS induces apoptosis *in vivo*, as the timing of the analysis might be critical and/or the cell population involved might be very small and difficult to assess by this type of analysis.

Task 3.3: Determine the effect of Bcl-xS on the growth of breast tumors *in vivo*

We have decided to mate WAP-Bcl-xS-HA transgenic mice with MMTV-erbB-2 transgenic mice (31) to assess the effect of Bcl-xS on the growth of breast tumors *in vivo*. MMTV-erbB-2 transgenic mice develop breast cancer at high frequency (31). In order to measure the effects of Bcl-xS on breast cancer developemnt *in vivo*, we needed to perform some preliminary experiments on the erb2B transgenic mice to assess the time course of the apperance of the breast cancers. In preliminary studies using a cohort of 12 MMTV-erbB-2 transgenic mice (breeding pairs obtained from Jackson Laboratories), we have evaluated in collaboration with Dr. Sofia Merajver (at our Institution), the development of breast tumors in WAP-erbB-2 transgenic mice. In our hands, MMTV-erbB-2 transgenic mice began to develop breast tumors by 150 days of age and by 240 days about 60% of mice have developed breast tumors (data not shown). We have mated male WAP-Bcl-xS-HA transgenic mice with female MMTV-erbB-2 transgenic mice to generate double WAP-Bcl-xS-HA/MMTV-erbB-2 transgenic mice and single transgenic mice as controls. These experiments that will determine the effect of the Bcl-xS transgene on breast cancer development should be completed within 1 year and are in progress.

KEY RESEARCH ACCOMPLISHMENTS

- The BH3 domain of Bcl-XS is required for apoptosis and binding to Bcl-XL
- Bcl-XS killing is mediated, at least in part, through the Apaf-1-caspase-9 pathway of apoptosis
- Bcl-XS binds Apaf-1 which appears to be mediated through Bcl-XL
- Identification and cloning of novel Apaf-1 cDNAs
- Expression and functional characterization of Apaf-1 isoforms
- Initial crossing of WAP-bclXS transgenic with MMTV-erb2B transgenic mice

REPORTABLE OUTCOMES

-Hu Y., Benedict MA, Ding L., and Núñez G. Role of Cytochrome c and dATP/ATP Hydrolysis in Apaf-1-Mediated Caspase-9 Activation and Apoptosis. *EMBO J.* 18: 3586-3595 (1999).

-Benedict MA., Hu Y., Inohara N., and Nuñez G. Expression and functional analysis of Apaf-1 Isoforms: Extra WD-40 repeat is required for cytochrome c binding and regulated activation of procaspase-9 (manuscript submitted for publication)

CONCLUSIONS

The studies that we have performed in the past year are important in that they have provided information regarding the mechanism by which the Bcl-xS kills tumor cells. The analysis showed that Bcl-xS kills cells, at least in part, through an Apaf-1-caspase-9 pathway. Bcl-xS associates with Bcl-xL via its BH3 domain and with Apaf-1 possibly through Bcl-xL. We have cloned and characterized a novel Apaf-1 cDNA form that, in contrast to the originally described Apaf-1, activates procaspase-9 in a cytochrome c and dATP-dependent manner. Finally, transgenic mice expressing Bcl-XS in the breast have been crossed with oncogenic erb2-B transgenic mice to assess the effects of Bcl-xS on breast cancer development.

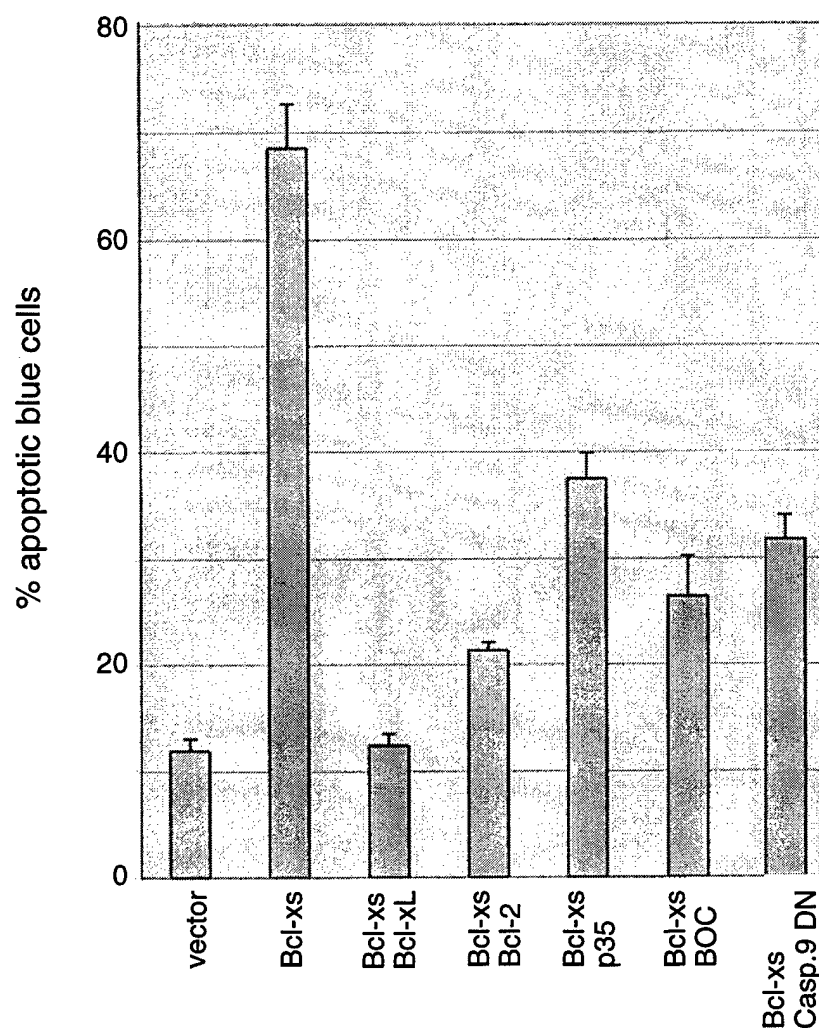
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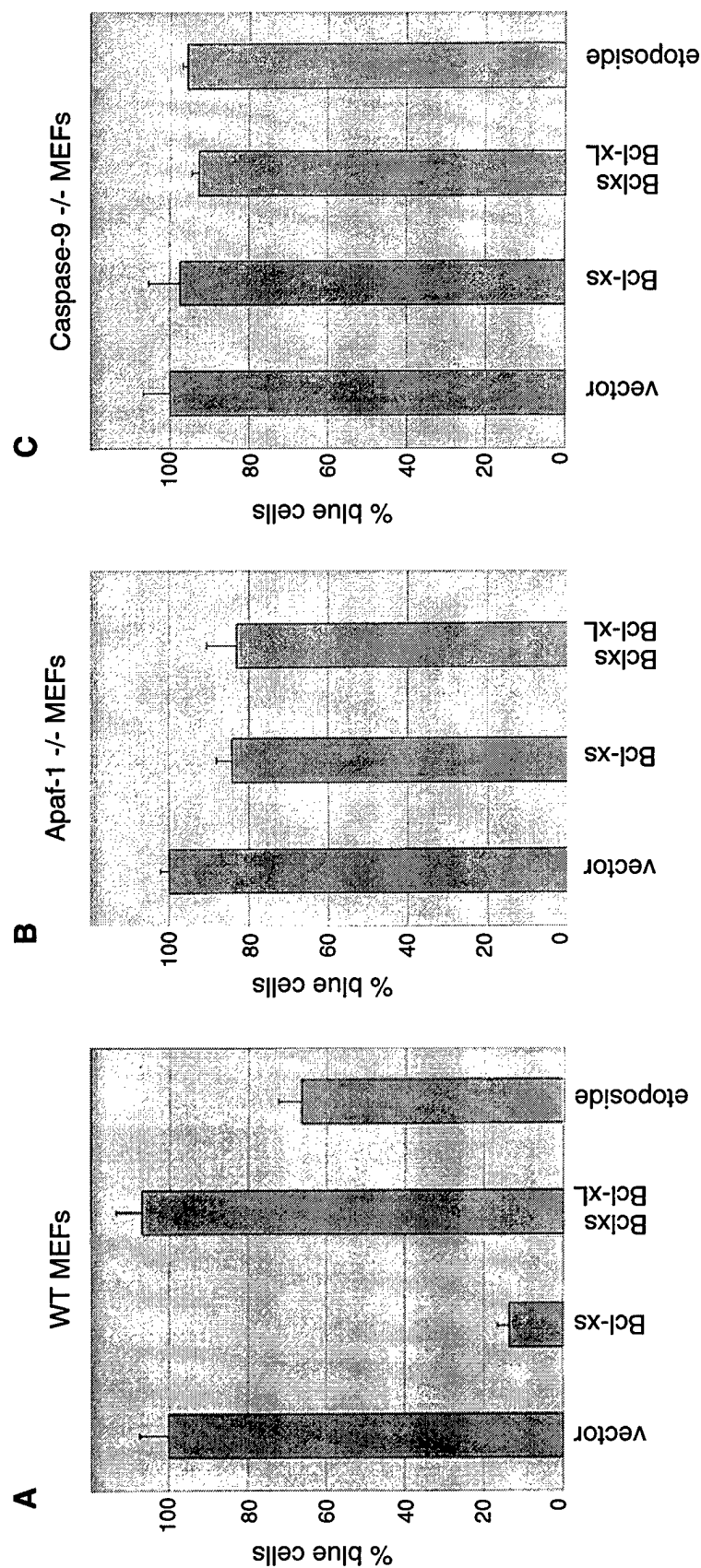
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APPENDIX



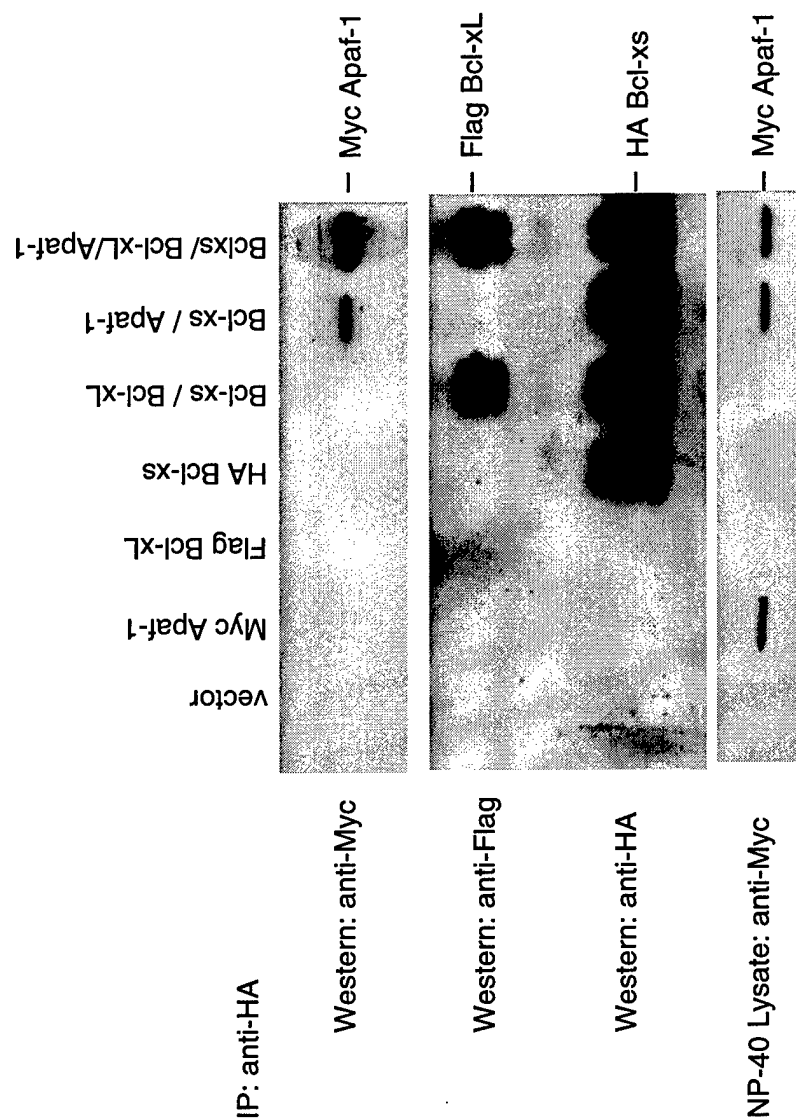
Bcl-xS killing is inhibited by Bcl-XL, Bcl-2 and a panel of caspase inhibitors.

Murine embryo fibroblasts were co-transfected in triplicate with Bgal, Bcl-xS and the indicated plasmids. In some experiments, transfected cells were incubated with the pan-caspase inhibitor, BOC-fmk. 16 hrs post-transfection, the number of blue cells with and without apoptotic morphology were counted. Data is expressed as % apoptotic blue cells of total number of blue cells per well, (average \pm SEM).



Bcl-xs killing requires both Apaf-1 and Caspase-9

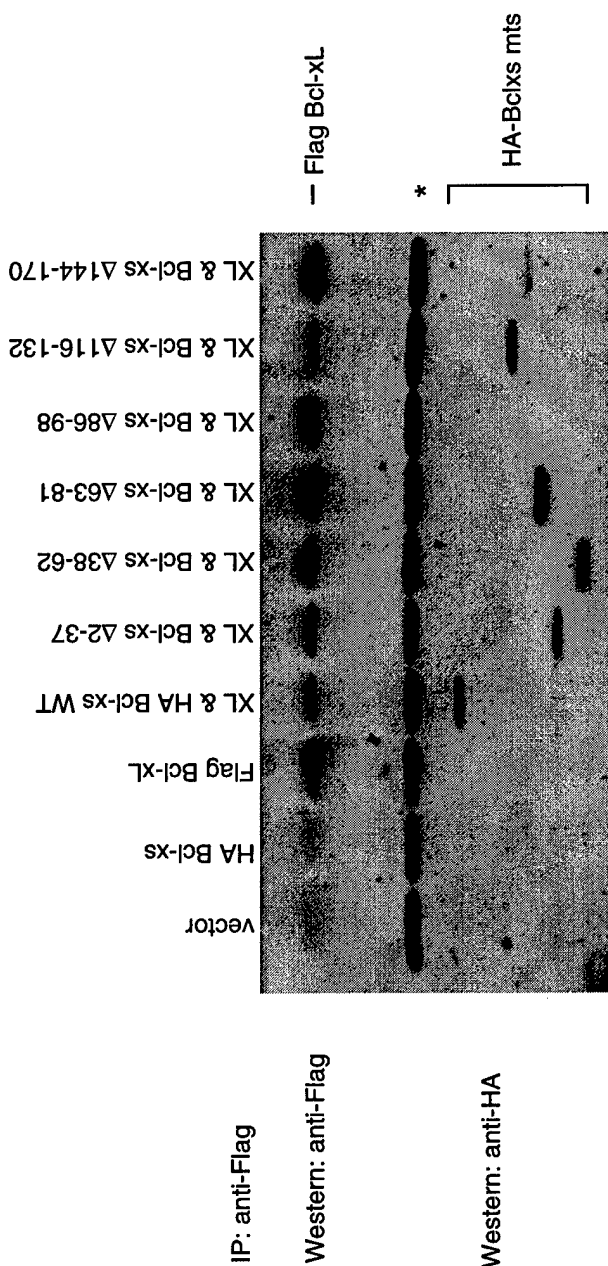
Murine embryo fibroblasts from wildtype (A), Apaf-1 knockout (B), and Caspase-9 knockout (C) mice were transiently co-transfected in triplicate with plasmids encoding Bgal and the empty vector, Bcl-xs, or Bcl-xs/Bcl-xL. Some vector/Bgal transfected cells were also treated with etoposide to confirm the phenotype of the knockout fibroblasts. 36 hrs post-transfection, cells were stained with X-gal, and the number of blue cells/well were counted. Data is expressed as % blue cells relative to the vector control (100%), (average \pm SEM).



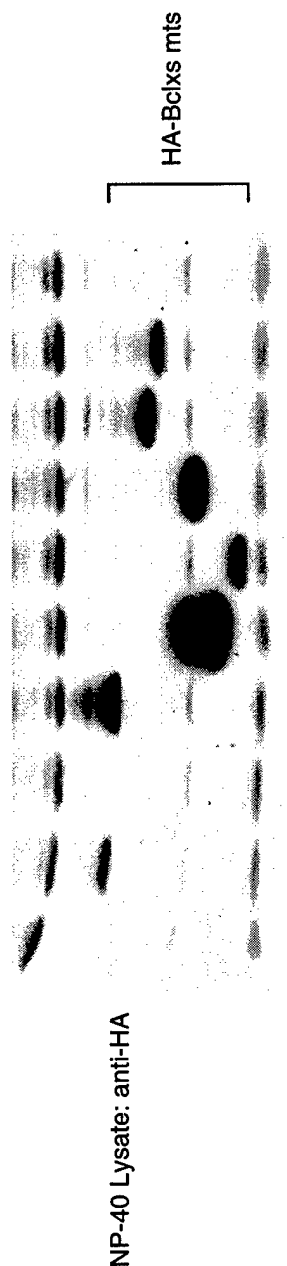
Bcl-Xs/Apaf-1 binding is increased by co-expression with Bcl-xL

Cancer cells were transiently transfected with plasmids expressing HA Bcl-xs, Flag Bcl-xL and Myc Apaf-1. Bcl-xs complexes were immunoprecipitated with anti-HA antibody and immunoblotted with anti-Flag, anti-HA, and anti-Myc. Upper panel shows Apaf-1 co-precipitated with Bcl-xs. Middle panel shows immunoprecipitated Bcl-xs and bound Bcl-xL. Lower panel shows equal levels of Apaf-1 in the NP-40 lysates used for immunoprecipitations.

A



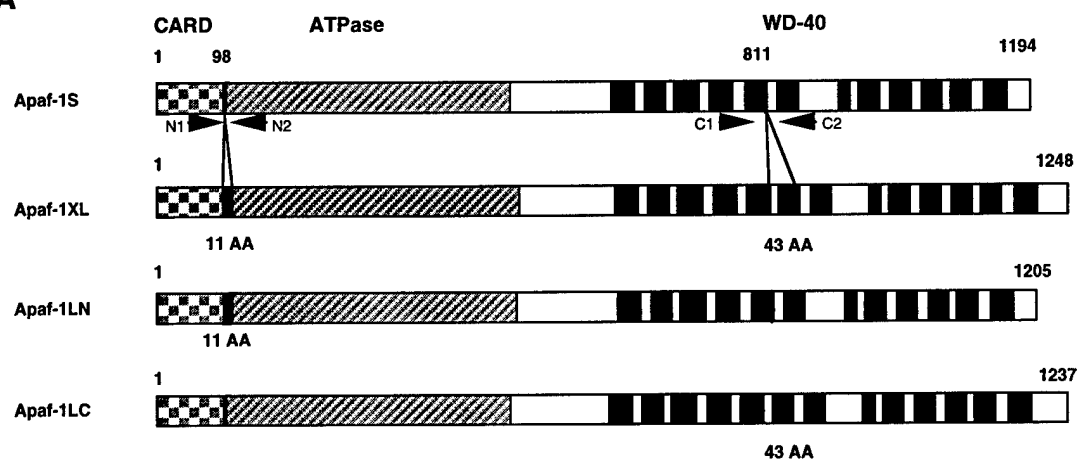
B



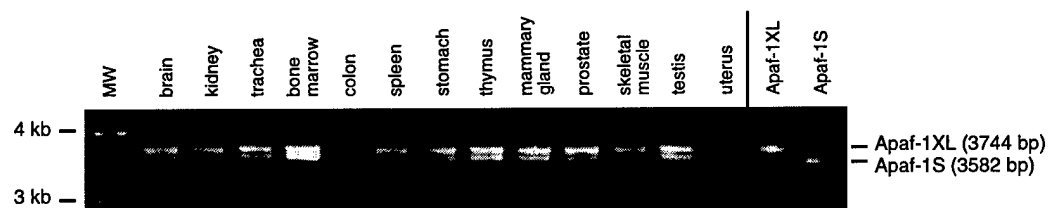
BH3 domain of Bcl-xS is required for binding to Bcl-xL

Cancer cells were transiently co-transfected with plasmids encoding Bcl-xL and Bcl-xS or Bcl-xS deletion mutants as indicated. Bcl-xS/ Bcl-xL complexes were immunoprecipitated with anti-Flag and immunoblotted with anti-HA and anti-Flag. Panel A shows immunoprecipitated Bcl-xL and bound Bcl-xS deletion mutants. * indicates non-specific IgG bands. Panel B shows an anti-HA immunoblot of NP-40 lysates used for the above immunoprecipitations, and demonstrates the expression of the Bcl-xS mutants. Note: Although deletion mutant Δ 144-170 is expressed (see Panel A, last lane), it is not visible on this exposure.

A



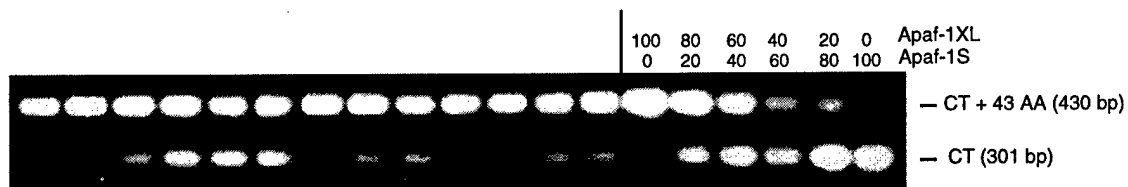
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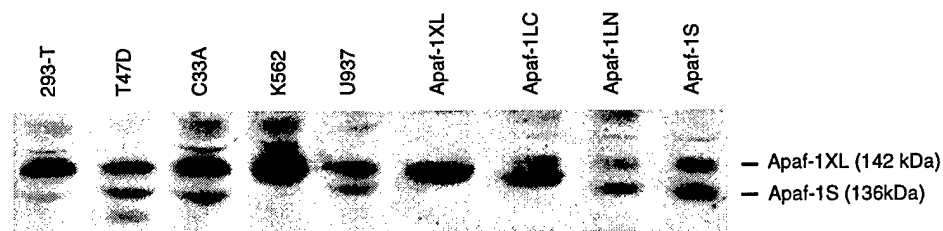
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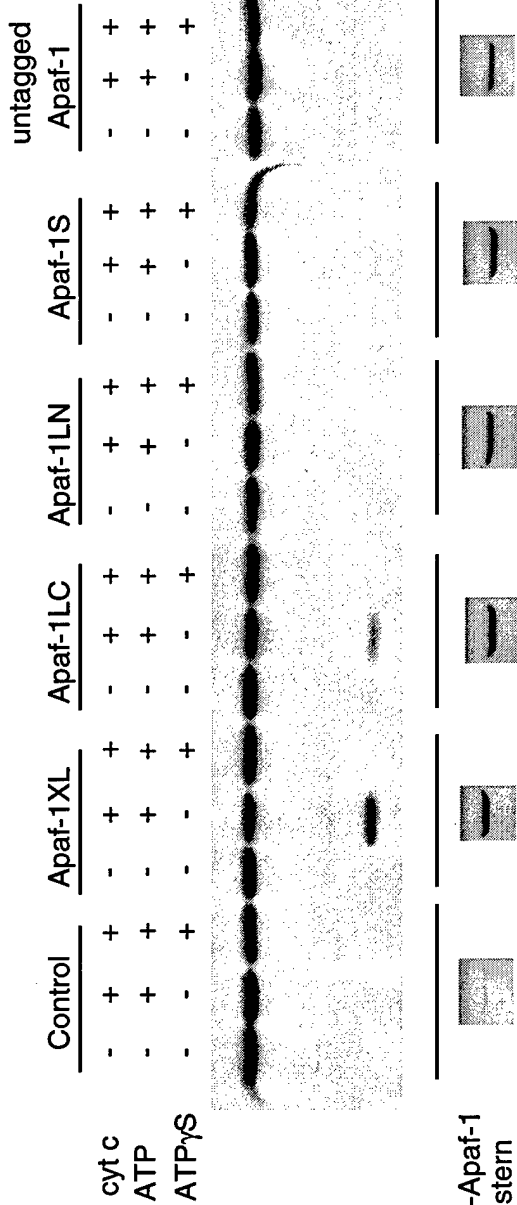


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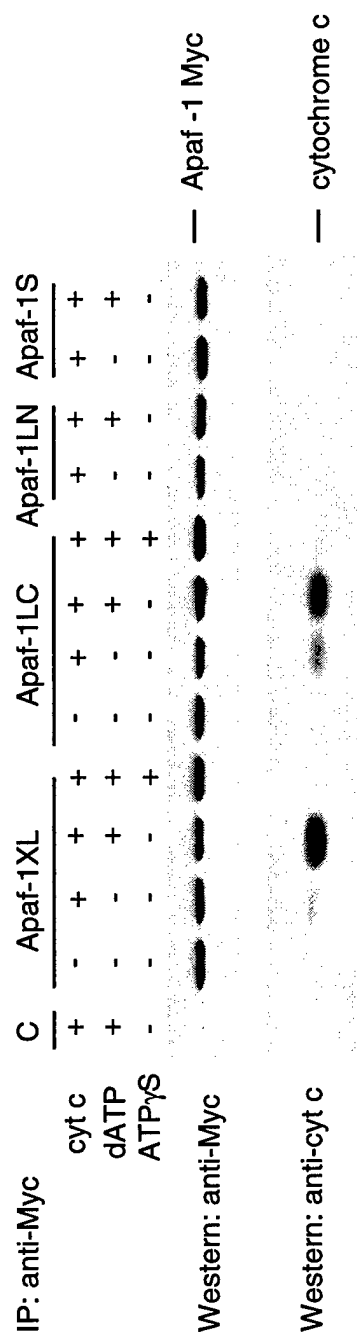


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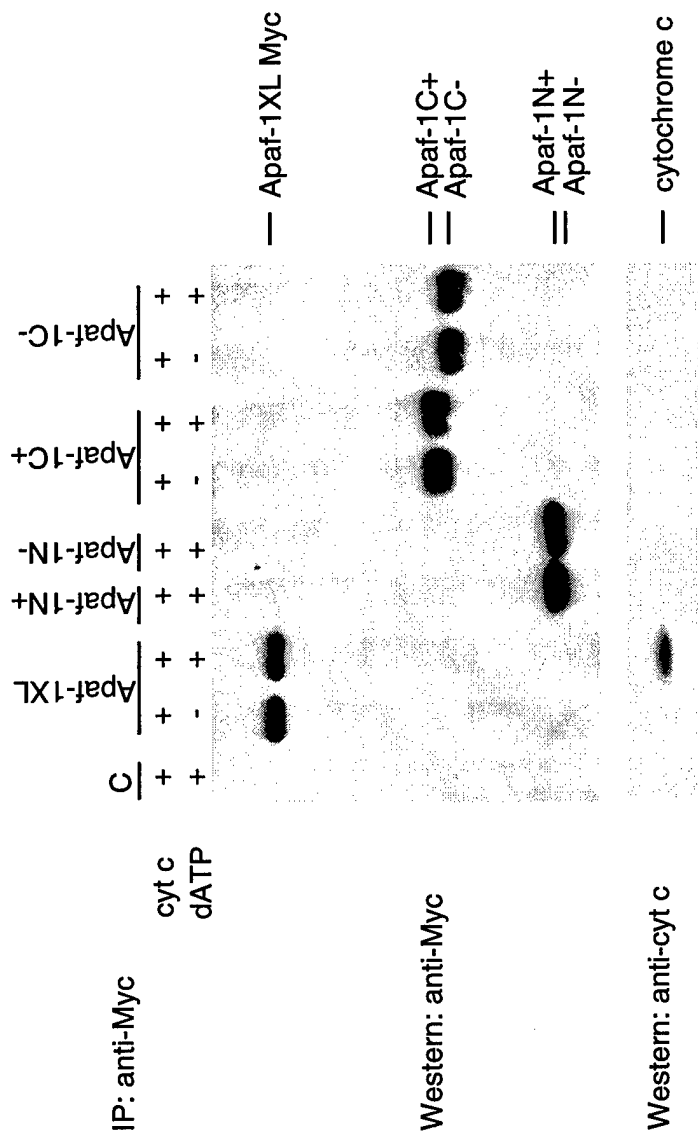




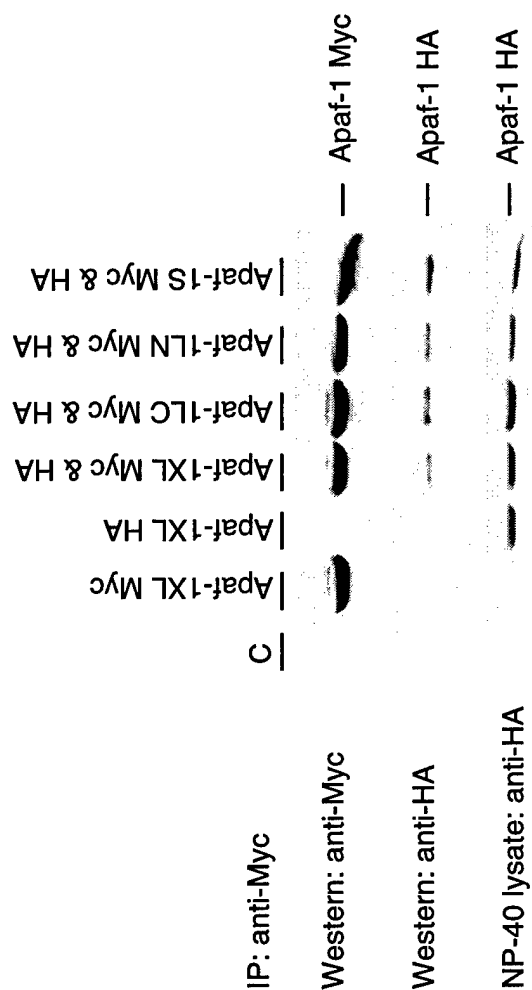
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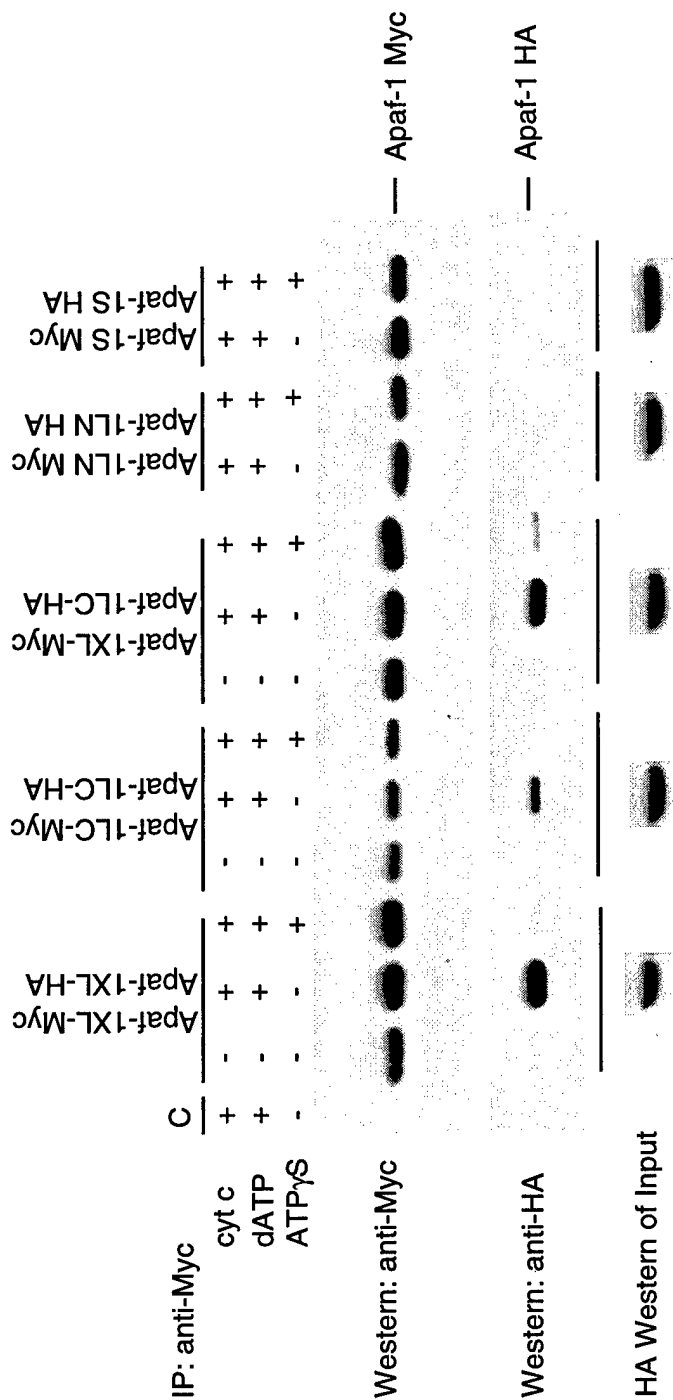
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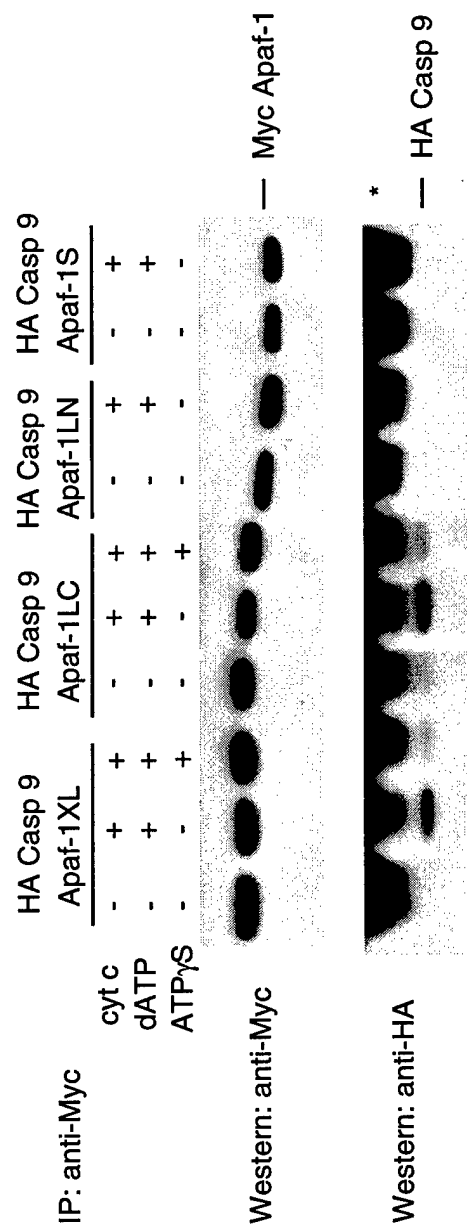
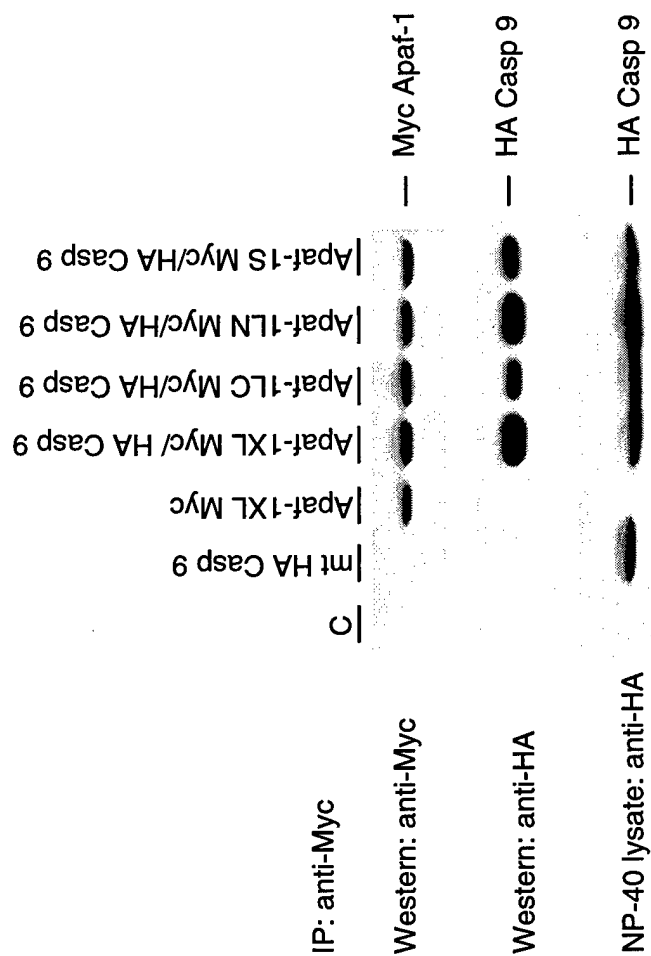
A



B



A



Legends for Figures 5-9

Fig. 5. Expression of Apaf-1 isoforms. A, schematic representation of Apaf-1 isoforms examined in this study. The caspase recruitment domain (CARD)1, ATPase domain, and WDRs are shown, as are the presence or absence of the 11 amino acid N-terminal insert following the CARD and the 43 amino acid C-terminal insert between the fifth and sixth WDRs. N1/N2 and C1/C2 represent the primers used to amplify the regions flanking the N-terminal and C-terminal inserts, respectively. The deletion mutant Apaf-1S (1-559) has been termed "N-", while the deletion mutant Apaf-1XL (1-570) has been termed "N+". "C-" refers to the deletion mutant Apaf-1S (468-1194), while "C+" refers to the deletion mutant Apaf-1XL (479-1248). B, RT-PCR analysis of the expression of full length Apaf-1 forms in human tissue RNAs. Primers used were identical to those used to amplify full length Apaf-1 cDNAs (see Experimental Procedures). The last two lanes are positive control reactions using either 10 pg of gel purified Apaf-1XL or Apaf-1S inserts as templates. C, RT-PCR analysis of the same human tissue RNAs as above, using primers N1 and N2. The last four lanes are positive control reactions in which the templates were 10 pg of Apf-1 XL and Apaf-1S, mixed at the indicated ratios. D, RT-PCR analysis of the human tissue RNAs using primers C1 and C2. The last six lanes are positive controls, with Apaf-XL and Apaf-1S mixed at the indicated ratios. E, Anti-Apaf-1 immunoblot analysis of 200 mg of cell lysate from various tumor cell lines. The last four lanes are positive control lysates from 293T cells transiently transfected with the indicated Apaf-1 plasmid. In addition to the transfected Apaf-1 isoforms, an endogenous Apaf-1 protein is observed in 293T cells.

Fig. 6. Cytochrome c-dependent *in vitro* activation of procaspase-9 requires the additional WD-40 repeat. Ten mg cytosolic extracts of 293T cells transiently transfected with either the vector control or the indicated Myc-tagged or untagged Apaf-1 isoforms were incubated with *in vitro* translated [35S] methionine-labeled procaspase-9, with or without 1 mM dATP, 8 mg/ml cytochrome c, or 5 mM ATP- γ -S at 30 °C for 30 min. Anti-Apaf-1 (X. Wang) immunoblot analysis of the cytosolic extracts used to measure *in vitro* procaspase-9 activation, is shown in the lower panel. Endogenous Apaf-1 protein, although present, is not detected in this exposure.

Fig. 7. The additional WD-40 repeat of Apaf-1 is necessary but not sufficient for the binding of cytochrome c. Cytosolic extracts from 293T cells transiently transfected with either vector control or the indicated Myc-tagged Apaf-1 plasmids were incubated with or without 8 mg/ml cytochrome c, 1 mM dATP and 5 mM ATP- γ -S in the presence of monoclonal anti-Myc antibody and protein A/G-agarose beads. After incubation for 2 h at 4°C, immunoprecipitation was performed as described in Experimental Procedures. Cytochrome c associated with Apaf-1 proteins was detected by immunoblotting. A, Immunoprecipitated full length Myc-

tagged Apaf-1 proteins are shown in the upper panel. Cytochrome c bound to Apaf-1 proteins is shown in the lower panel. B, Immunoprecipitated Myc-Apaf-1XL and Myc-tagged Apaf-1 deletion mutants are shown in the upper panel. Bound cytochrome c is shown in the lower panel.

Fig. 8. Cytochrome c/dATP-dependent Apaf-1 Self-association requires the additional WD-40 repeat. A, In NP-40 extracts, all Apaf-1 isoforms can self-associate. NP-40 extracts of 293T cells transiently transfected with the indicated Myc- or HA-tagged Apaf-1 plasmids were immunoprecipitated with rabbit anti-Myc antibody and immune complexes were immunoblotted with either mouse anti-Myc or mouse anti-HA. Immunoprecipitated Myc-Apaf-1 proteins are shown in the upper panel, and self-associated HA-Apaf-1 proteins are shown in the middle panel. The lower panel depicts an anti-HA immunoblot of the NP-40 extracts to confirm equivalent expression of the HA-Apaf-1 isoforms used. B, In cytosolic extracts, cytochrome c/dATP-dependent Apaf-1 Self-association requires the additional WD-40 repeat. 293T cells were transiently transfected with the indicated Myc- or HA-tagged Apaf-1 plasmid. Cytosolic extracts of the indicated plasmids were combined with or without 8 mg/ml cytochrome c, 1 mM dATP and 5 mM ATP-g-S, in the presence of polyclonal anti-Myc antibody and protein A/G-agarose beads. Immunoprecipitation and anti- Myc or -HA immunoblotting was performed as described above. Immunoprecipitated Myc-Apaf-1 isoforms are shown in the upper panel, and associated HA-Apaf-1 isoforms are shown in the middle panel. The lower panel depicts an anti-HA immunoblot of the cytosolic extracts to confirm equivalent expression of the HA-Apaf-1 isoforms used.

Fig. 9. Cytochrome c-dependent binding of Apaf-1 to procaspase-9 requires the extra WD-40 repeat. A, In NP-40 extracts, all Apaf-1 isoforms can bind to procaspase-9. NP-40 extracts of 293T cells transiently co-transfected with the indicated Myc- Apaf-1 plasmids and HA-mt procaspase-9(C287S) were immunoprecipitated with rabbit anti-Myc antibody and immune complexes were immunoblotted with either mouse anti-Myc or mouse anti-HA. Immunoprecipitated Myc Apaf-1 isoforms are shown in the upper panel, and associated HA-mt procaspase-9 is shown in the middle panel. The lower panel depicts an anti-HA immunoblot of the NP-40 extracts to confirm similar expression of HA-mt procaspase-9 in each extract. B, In cytosolic extracts, cytochrome c/dATP-dependent binding of Apaf-1 to procaspase-9 requires the additional WDR. 293T cells were transiently transfected with the indicated Myc-Apaf-1 plasmid or HA-mt procaspase-9. Cytosolic extracts of the indicated plasmids were combined with or without 8 mg/ml cytochrome c, 1 mM dATP and 5 mM ATP-g-S, in the presence of polyclonal anti-Myc antibody and protein A/G-agarose beads. Immunoprecipitation and anti- Myc or -HA immunoblotting was performed as described above. Immunoprecipitated Myc-Apaf-1 isoforms are shown in the upper panel and associated HA-mt procaspase-9 is shown in the bottom panel. The asterisk denotes non-specific IgG heavy chain bands.

Role of cytochrome *c* and dATP/ATP hydrolysis in Apaf-1-mediated caspase-9 activation and apoptosis

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Apaf-1 plays a critical role in apoptosis by binding to and activating procaspase-9. We have identified a novel Apaf-1 cDNA encoding a protein of 1248 amino acids containing an insertion of 11 residues between the CARD and ATPase domains, and another 43 amino acid insertion creating an additional WD-40 repeat. The product of this Apaf-1 cDNA activated procaspase-9 in a cytochrome *c* and dATP/ATP-dependent manner. We used this Apaf-1 to show that Apaf-1 requires dATP/ATP hydrolysis to interact with cytochrome *c*, self-associate and bind to procaspase-9. A P-loop mutant (Apaf-1K160R) was unable to associate with Apaf-1 or bind to procaspase-9. Mutation of Met368 to Leu enabled Apaf-1 to self-associate and bind procaspase-9 independent of cytochrome *c*, though still requiring dATP/ATP for these activities. The Apaf-1M368L mutant exhibited greater ability to induce apoptosis compared with the wild-type Apaf-1. We also show that procaspase-9 can recruit procaspase-3 to the Apaf-1-procaspase-9 complex. Apaf-1(1-570), a mutant lacking the WD-40 repeats, associated with and activated procaspase-9, but failed to recruit procaspase-3 and induce apoptosis. These results suggest that the WD-40 repeats may be involved in procaspase-9-mediated procaspase-3 recruitment. These studies elucidate biochemical steps required for Apaf-1 to activate procaspase-9 and induce apoptosis.
Keywords: Apaf-1/apoptosis/caspase-3/caspase-9

Introduction

Programmed cell death, or apoptosis, an evolutionarily conserved and genetically regulated biological process, plays a critical role in the development and the regulation of tissue homeostasis of multicellular organisms (Thompson, 1995; Yuan, 1996). Genetic analyses of the nematode *Caenorhabditis elegans* have identified two genes, *ced-3* and *ced-4*, required for the execution of cell death in the worm (Hengartner and Horvitz, 1994). The product of *ced-3* is homologous to mammalian interleukin-1 β -converting enzyme, a finding that provided the first indication that cysteine proteases are critical components of the cell death machinery (Miura *et al.*, 1993). These observations led to the identification of a growing family of cysteine proteases (designated as

caspases) related to CED-3 that represent the executionary arm of the apoptotic program in mammals, flies and nematodes (Núñez *et al.*, 1998; Thornberry and Lazebnik, 1998). Caspases are synthesized in cells as inactive precursors which upon stimulation with apoptotic signals are processed into mature forms composed of a tetramer of two large and two small subunits (Thornberry *et al.*, 1992; Walker *et al.*, 1994; Rotonda *et al.*, 1996). The apoptotic process is characterized by the proteolytic cascade in which upstream (initiator) caspases mediate the activation of downstream (effector) caspases (Alnemri, 1997; Núñez *et al.*, 1998). Initiator caspases contain death effector domains (DEDs) or caspase recruitment domains (CARDs) that physically link these proteases to regulatory molecules via homophilic interactions (Li *et al.*, 1997; Pan *et al.*, 1998). Caspase-9 is an upstream caspase that contains a CARD in its N-terminus; a protein module that is also present in the prodomain of several death proteases, including CED-3 and its regulator CED-4 (Hofmann *et al.*, 1997). Activation of caspase-9 initiates a protease cascade that subsequently activates downstream caspase-3 and caspase-7, leading to the cleavage of target proteins and the orderly demise of the cell (Li *et al.*, 1997; Sun *et al.*, 1999).

Our understanding of how caspases are activated in mammalian cells has been greatly enhanced by biochemical studies of the nematode proteins CED-3, CED-4 and CED-9. CED-4 physically interacts with both CED-3 and CED-9, forming a multimeric protein complex (Chinnaiyan *et al.*, 1997; Irmeler *et al.*, 1997; James *et al.*, 1997; Spector *et al.*, 1997; Wu *et al.*, 1997a,b). CED-4 promotes the activation of CED-3 and this activation process is inhibited by CED-9 (Seshagiri and Miller, 1997; Wu *et al.*, 1997b). Biochemical experiments have suggested that a mechanism exists whereby CED-3 is activated through oligomerization of CED-4 (Yang *et al.*, 1998). Activation of CED-3 is induced through aggregation of precursor CED-3 molecules, an event leading to CED-3 autoprocessing and enzymatic activation (Yang *et al.*, 1998).

Apaf-1, a mammalian homologue of CED-4, has been identified recently (Zou *et al.*, 1997; Fearnhead *et al.*, 1998). The N-terminal half of Apaf-1 shares extensive homology with CED-4, containing a CARD followed by an ATPase domain with conserved Walker's A and B motifs (Zou *et al.*, 1997). The C-terminal region of Apaf-1 lacks homology with CED-4 and consists of 12 WD-40 repeats. An important role for Apaf-1 in the regulation of apoptosis has been revealed by analysis of mutant mice deficient in Apaf-1. Mice lacking Apaf-1 showed abnormalities in several tissues characterized by the lack of developmental cell death, particularly in brain tissue (Cecconi *et al.*, 1998; Yoshida *et al.*, 1998). Furthermore, cells derived from Apaf-1-deficient mice showed resist-

ance to a wide variety of apoptotic stimuli, including chemotherapy drugs, dexamethasone and γ -irradiation (Yoshida *et al.*, 1998). In the presence of cytochrome *c* and dATP, Apaf-1 adopts a conformation that can bind to procaspase-9, an event that leads to its proteolytic activation (Li *et al.*, 1997). A conserved mechanism similar to that proposed for CED-3 has been suggested for caspase-9 activation (Hu *et al.*, 1998b; Srinivasula *et al.*, 1998). In this model, two or more caspase-9 precursors are brought together via Apaf-1 oligomerization, which is mediated by the ATPase-like domain of Apaf-1 (Hu *et al.*, 1998b; Srinivasula *et al.*, 1998). Mutant analyses of Apaf-1 have suggested that the WD-40 repeat region (WDR) plays an inhibitory role in Apaf-1 function, since mutants lacking the WDR are constitutively active (Hu *et al.*, 1998a,b; Srinivasula *et al.*, 1998). Furthermore, the WDR has been shown to interact with the ATPase-like domain of Apaf-1, inhibiting Apaf-1 self-association. This finding suggests the existence of a mechanism whereby the WDR inhibits Apaf-1 activity (Hu *et al.*, 1998b).

In these studies, we sought to understand further the biochemical mechanism by which Apaf-1 promotes the maturation of procaspase-9 into an active protease. Analysis of wild-type (wt) and mutant Apaf-1 proteins revealed that dATP/ATP hydrolysis is required for cytochrome *c* binding to Apaf-1, Apaf-1 self-association, interaction of Apaf-1 with procaspase-9, and ultimately for caspase-9 activation and caspase-9-mediated apoptosis. We also provide evidence that the Apaf-1-caspase-9 complex recruits procaspase-3, a process that may be regulated by the WDR of Apaf-1.

Results

Identification of Apaf-1XL, an alternatively spliced Apaf-1 cDNA that activates procaspase-9

In our initial studies of Apaf-1-mediated caspase-9 activation, we determined that procaspase-9 could be efficiently activated by endogenous Apaf-1 from several cell lines including HeLa and 293T cells (Hu *et al.*, 1998a; data not shown). We subsequently identified Apaf-1 cDNAs from these two cell lines. An Apaf-1 cDNA cloned from HeLa cells was identical to that previously reported (Zou *et al.*, 1997). However, the Apaf-1 cDNA cloned from 293T cells appeared to represent an alternatively spliced form of the Apaf-1 gene. This form, termed Apaf-1XL, contains an 11 amino acid (aa) insertion, GKDSVSGITSY, after the CARD (inserted between amino acids 98 and 99), and another 43 aa insertion between amino acids 811 and 812 (Figure 1A). This 43 aa region contains the last three amino acids of a WD-40 repeat followed by a large portion of another WD-40 repeat, thus creating an additional WD-40 repeat (Figure 1A). The sequence of these extra 43 aa is identical to that present in a truncated human Apaf-1 cDNA submitted to databases (DDBJ/EMBL/GenBank accession No. AB007873). Moreover, a mouse Apaf-1 cDNA isolated from brain tissue also contained this additional WD-40 repeat (Cecconi *et al.*, 1998). In addition, a recent study has identified an Apaf-1 form in HeLa cells that is identical to Apaf-1XL (Zou *et al.*, 1999). Using reverse transcriptase (RT)-PCR and Western blotting analysis, we have identified both Apaf-1XL mRNA and protein in 16 cell lines tested so far (data

not shown). Under our experimental conditions, Apaf-1XL protein expressed in 293T cells activated procaspase-9 in a cytochrome *c*- and dATP-dependent manner (Figure 1D). Under the same conditions, we were unable to demonstrate cytochrome *c*- and dATP-dependent procaspase-9 activation by the product of the original Apaf-1 cDNA (data not shown). Therefore, in the present studies we used the Apaf-1XL cDNA to examine the mechanism by which Apaf-1 activates procaspase-9 and refer to it as Apaf-1 for simplicity.

Mutation of Met368 to Leu enables full-length Apaf-1 to activate procaspase-9 independently of cytochrome *c* and dATP

We demonstrated previously that the WDR interacts with the N-terminal CED-4 homologous region of Apaf-1 and inhibits procaspase-9 activation when co-expressed with the constitutively active N-terminal Apaf-1 mutants (Hu *et al.*, 1998b). We performed experiments to determine whether the WDR with an extra WD-40 repeat is also capable of binding to N-terminal Apaf-1 with an 11 aa insertion [Apaf-1(1-570)] and inhibiting procaspase-9 activation. The analyses showed that the WDR containing the extra WD-40 repeat associated with Apaf-1(1-570) (Figure 1B) and inhibited procaspase-9 activation promoted by Apaf-1(1-570) (Figure 1C). To understand further how Apaf-1 promotes procaspase-9 activation, we tested several Apaf-1 N-terminal mutants accumulated during PCR mutagenesis for their ability to interact with the WDR. A mutant substituting amino acid Met368 for Leu, Apaf-1(1-570M368L), showed a reduced ability to bind the WDR (Figure 1B), but it retained its ability to promote procaspase-9 activation independent of cytochrome *c* and dATP (Figure 1C). In contrast to Apaf-1(1-570), however, the ability of Apaf-1(1-570M368L) to induce procaspase-9 activation was not inhibited by the WDR (Figure 1C). We next incorporated the M368L point mutation into full-length Apaf-1 and determined the ability of wt and mutant Apaf-1 to activate procaspase-9. In these experiments, we prepared cell extracts from 293T cells transiently transfected with Apaf-1 constructs and diluted the extracts so that endogenous Apaf-1 activity could not be detected in the procaspase-9 activation assay. Full-length Apaf-1 induced activation of procaspase-9 in a dATP- and cytochrome *c*-dependent manner (Figure 1D). In contrast, the point mutant Apaf-1M368L activated procaspase-9 independent of cytochrome *c* and dATP (Figure 1D). Apaf-1K160R, a mutant substituting Lys160 [an essential amino acid residue in the phosphate-binding loop (P-loop)] for Arg, failed to activate procaspase-9 in the presence or absence of dATP and cytochrome *c* (Figure 1D). This result confirmed previous observations that dATP/ATP hydrolysis is required for Apaf-1 to induce procaspase-9 activation (Li *et al.*, 1997).

The cytochrome *c*-Apaf-1 interaction requires ATP hydrolysis

To determine the role for cytochrome *c* in procaspase-9 activation, we immunoprecipitated wt and mutant Apaf-1 from cytosolic extracts in the presence or absence of dATP and assessed the binding of cytochrome *c* to Apaf-1 proteins by immunoblotting. Cytochrome *c* bound to

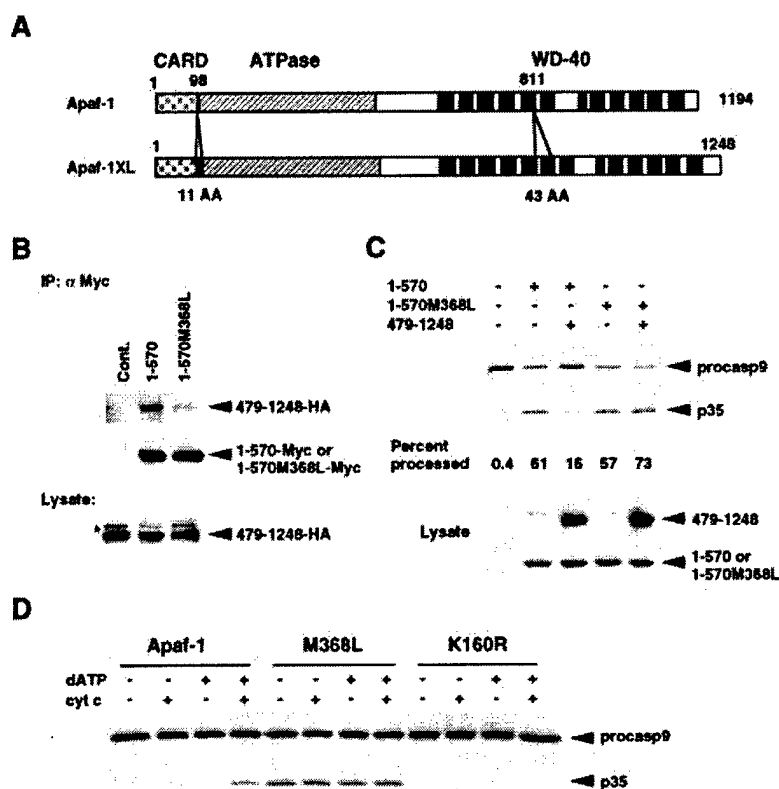


Fig. 1. Mutation of Met368 to Leu renders Apaf-1 capable of activating procaspase-9 independent of cytochrome *c* and dATP. (A) Schematic representation of the original Apaf-1 and Apaf-1XL. The 11 amino acids inserted after aa 98 are GKDSVSGITSY. The sequence of the extra 43 aa was identical to that identified in an Apaf-1 cDNA that is deposited in the DDBJ/EMBL/GenBank database (accession No. AB007873). (B) Mutation of Met368 to Leu decreases binding of Apaf-1(1-570) to WDR (WD-40 repeat region). pcDNA3 or plasmids encoding Myc-tagged Apaf-1(1-570) or Apaf-1(1-570M368L) were transfected into 293T cells with a construct producing HA-tagged WDR (aa 479-1248). Total cell lysates were immunoprecipitated with rabbit anti-c-Myc antibody and immune complexes were immunoblotted with anti-HA to detect the WDR. The asterisk indicates a non-specific band. (C) WDR fails to inhibit Apaf-1(1-570M368L)-mediated procaspase-9 activation. Twenty micrograms of cytosolic extracts containing indicated Apaf-1 mutants were incubated with *in vitro* translated [³⁵S]methionine-labeled procaspase-9 at 30°C for 30 min and separated by SDS-PAGE. Immature and cleaved caspase-9 fragments were scanned and quantitated with a phosphorimager and normalized according to the numbers of methionine residues in each form. Percentage of procaspase-9 converted into p35 is shown below each lane. Lower panel shows equal amounts of Apaf-1 mutant proteins in extracts. (D) Mutation of Met368 to Leu renders full-length Apaf-1 capable of activating procaspase-9 independent of cytochrome *c* and dATP. Two micrograms of extracts containing Apaf-1, Apaf-1M368L, or Apaf-1K160R were incubated with or without 1 mM dATP and 0.2 μ g of cytochrome *c* at 30°C for 30 min.

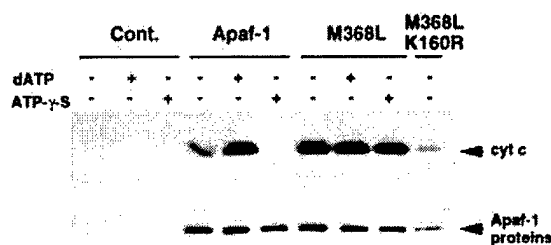


Fig. 2. Binding of cytochrome *c* to Apaf-1 requires dATP hydrolysis. Cytosolic extracts containing Myc-tagged Apaf-1, Apaf-1M368L or Apaf-1M368L/K160R were incubated with 10 μ g/ml cytochrome *c* with or without 1 mM dATP or ATP- γ S in the presence of monoclonal anti-Myc and protein A/G-agarose beads. After incubation at 4°C for 2 h, immunoprecipitation was performed as described in Materials and methods and cytochrome *c* associated with Apaf-1 proteins detected by immunoblotting. Wt and mutant Apaf-1 proteins precipitated from cytosolic extracts are shown in the lower panel.

Apaf-1, and this binding was greatly enhanced by the presence of dATP in the extract. Incubation of the extracts with the non-hydrolyzable ATP- γ S completely abolished cytochrome *c* binding to wt Apaf-1, indicating that dATP/ATP hydrolysis is essential for Apaf-1 to interact with cytochrome *c* (Figure 2). The weak binding of

cytochrome *c* to Apaf-1 detected in the absence of dATP is presumably due to low levels of endogenous dATP/ATP in the cell extract. In contrast, cytochrome *c* interacted with Apaf-1M368L in the presence or absence of dATP (Figure 2). Addition of ATP- γ S failed to inhibit cytochrome *c* binding to mutant Apaf-1M368L (Figure 2). Mutation of Lys160 (the critical P-loop residue required for ATP binding/hydrolysis) to Arg did not abolish the binding of Apaf-1M368L to cytochrome *c* (Figure 2), providing further evidence that cytochrome *c* can bind to Apaf-1M368L independent of dATP binding and hydrolysis. The diminished binding of cytochrome *c* to the double mutant Apaf-1M368L/K160R, when compared with wt Apaf-1 or Apaf-1M368L, could be explained by reduced expression of the double mutant Apaf-1 protein in the extract (Figure 2).

Apaf-1 self-association requires dATP/ATP hydrolysis

It was previously reported that Apaf-1 requires dATP/ATP to activate procaspase-9 (Li *et al.*, 1997). dATP/ATP hydrolysis could be required for Apaf-1 self-association and/or the Apaf-1-procaspase-9 interaction, since a disruption

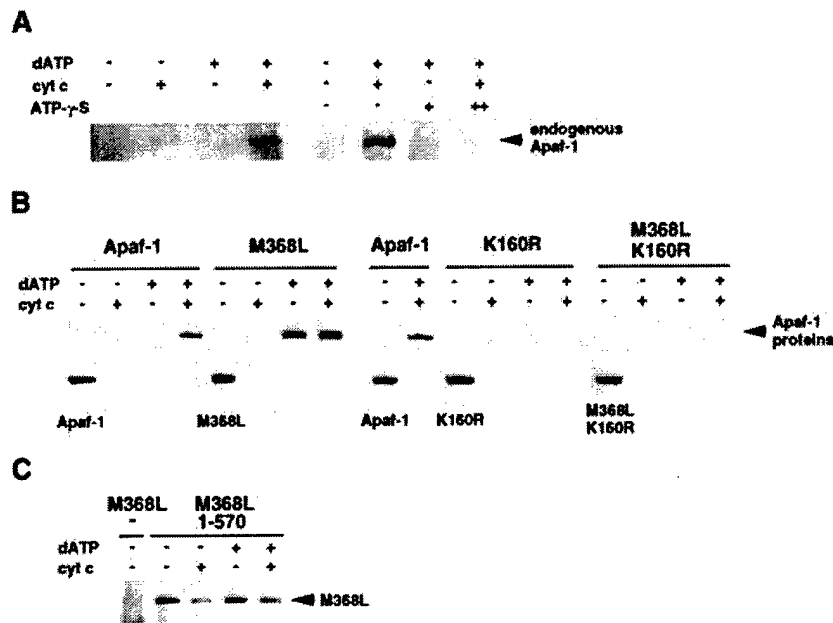


Fig. 3. Apaf-1 self-association requires cytochrome *c* and dATP hydrolysis. (A) Endogenous Apaf-1 binds Apaf-1(1–570) in the presence of cytochrome *c* and dATP. Equal amounts of 293T cytosolic extracts containing Myc-tagged Apaf-1(1–570) were incubated with rabbit anti-Myc antibody and protein A/G-agarose beads (1:1) under the indicated conditions at 4°C for 2 h. Then the beads were pelleted and washed four times with 1 ml NP-40 buffer. '11' indicates that 5 mM ATP-γS was included in the extract. (B) Interaction of wt Apaf-1, Apaf-1M368L, Apaf-1K160R, or Apaf-1M368L/K160R with Apaf-1(1–570). The experiments were performed as in (A) except that Apaf-1(1–570)-FLAG-containing extracts were mixed with lysates containing Apaf-1, Apaf-1M368L, Apaf-1K160R, or Apaf-1M368L/K160R. Expression of wt and mutant Apaf-1 proteins in total lysates is shown in lower panels. (C) Apaf-1M368L can associate with Apaf-1(1–570) independent of cytochrome *c* and dATP. Constructs producing Apaf-1(1–570) and Apaf-1M368L were cotransfected into 293T cells and prepared total lysates were used for immunoprecipitation as described in (A).

tion of either step might be sufficient to inhibit procaspase-9 activation (Hu *et al.*, 1998b; Srinivasula *et al.*, 1998). We first determined whether dATP/ATP hydrolysis is required for Apaf-1 self-association in cytosolic extracts. We transiently transfected 293T cells with a Myc-tagged Apaf-1(1–570) construct and assessed its interaction with endogenous Apaf-1 in the absence of detergent. Immunoprecipitation experiments showed that endogenous Apaf-1 associated with Apaf-1(1–570) in the presence of cytochrome *c* and dATP, but not in the absence of these factors (Figure 3A). The non-hydrolyzable ATP analogue, ATP-γS, failed to substitute for dATP (Figure 3A), indicating that ATP hydrolysis rather than simple ATP binding is required for Apaf-1 to self-associate. We next prepared extracts from cells transfected with wt and mutant Apaf-1 constructs to analyze further the requirement of dATP and/or cytochrome *c* for Apaf-1 self-association. Immunoprecipitation analysis showed that Apaf-1 self-association required both cytochrome *c* and dATP (Figure 3B), in agreement with the results obtained with endogenous Apaf-1. The constitutively active Apaf-1M368L mutant associated with Apaf-1 in the absence of cytochrome *c*, but required dATP for association with Apaf-1(1–570) (Figure 3B). Mutation of the critical P-loop Lys160 to Arg (M368L/K160R) abolished the ability of Apaf-1M368L to associate with Apaf-1 (Figure 3B). When co-expressed with Apaf-1(1–570), Apaf-1M368L interacted with Apaf-1(1–570) without the addition of exogenous dATP (Figure 3C), suggesting that intracellular dATP/ATP can promote their association.

Procaspase-9 binding to Apaf-1 requires dATP/ATP hydrolysis

To examine the role of dATP/ATP hydrolysis in procaspase-9 binding, we first determined whether dATP/

ATP binding/hydrolysis is required for endogenous Apaf-1 to bind procaspase-9 in cytosolic extracts. We began by transiently transfecting 293T cells with hemagglutinin (HA)-tagged procaspase-9 (C287S) and assessing the interaction of procaspase-9 with endogenous Apaf-1 in the absence of detergent. Immunoprecipitation of procaspase-9 showed that endogenous Apaf-1 binds to procaspase-9 in the presence of cytochrome *c* and dATP, but not in the absence of these factors (Figure 4A). In addition, the non-hydrolyzable ATP analogue, ATP-γS, inhibited procaspase-9 binding to endogenous Apaf-1 (Figure 4A), indicating that ATP hydrolysis is required for Apaf-1 to bind procaspase-9. We next performed immunoprecipitations with cytosolic extracts from cells transfected with wt and mutant Apaf-1 constructs to further dissect the requirement of dATP and/or cytochrome *c* for the binding of procaspase-9 to Apaf-1. To avoid the formation of Apaf-1 protein complexes in living cells, we prepared cellular extracts expressing wt or mutant Apaf-1 and incubated them with separately prepared extracts containing procaspase-9 (C287S). As we showed with endogenous Apaf-1, exogenous Apaf-1 required both cytochrome *c* and dATP to interact with procaspase-9 (Figure 4B). Mutation of Lys160 to Arg abolished procaspase-9 binding to Apaf-1, confirming that dATP/ATP hydrolysis is needed for the Apaf-1–procaspase-9 interaction. The constitutively active Apaf-1M368L mutant interacted with procaspase-9 in the absence of dATP and cytochrome *c* (Figure 4B), which is consistent with the observation that this mutant does not require these factors for activation of procaspase-9. Furthermore, in contrast to endogenous Apaf-1, incubation with ATP-γS did not inhibit the binding of mutant Apaf-1M368L to procaspase-9 (data not shown). Surprisingly, mutation of

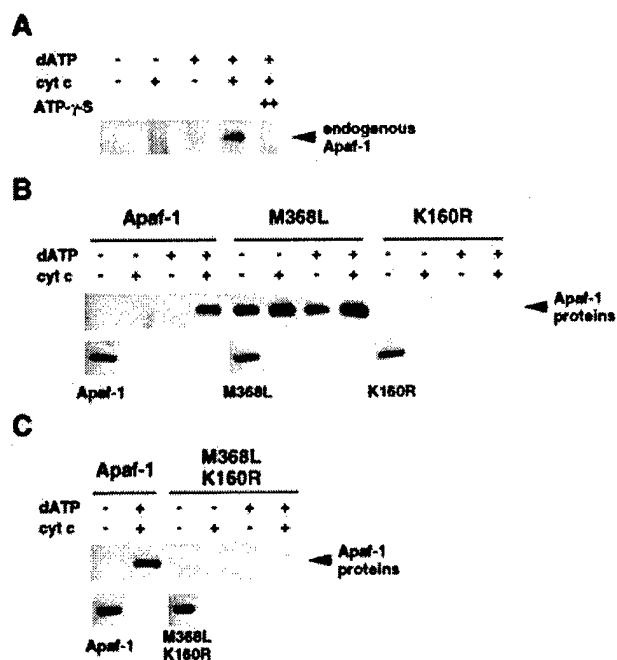


Fig. 4. Cytochrome *c* and ATP hydrolysis are required for Apaf-1 to interact with procaspase-9. (A) Procaspase-9 interacts with endogenous Apaf-1 in a cytochrome *c* and dATP-dependent manner. Equal amounts of 293T cytosolic extracts containing procaspase-9 (C287S)-HA were incubated with rabbit anti-HA antibody and protein A/G-agarose beads (1:1) under the indicated conditions at 4°C for 2 h. Then the beads were pelleted and washed four times with 1 ml NP-40 buffer. '11' indicates that 5 mM ATP- γ S was included in the extract. (B) Procaspase-9 interacts with Apaf-1M368L in the absence of cytochrome *c* and dATP. The experiments were performed as in (A) except that procaspase-9(C287S)-HA-containing extracts were mixed with lysates containing Apaf-1, Apaf-1M368L or Apaf-1K160R. (C) A double-point mutant, Apaf-1M368L/K160R fails to interact with procaspase-9. Expression of wt and mutant Apaf-1 proteins in total lysates is shown in the lower panels in (B) and (C).

Lys160 to Arg abolished the ability of Apaf-1M368L to interact with procaspase-9 (Figure 4C), suggesting that dATP/ATP binding/hydrolysis is indeed required for this point mutant to associate with procaspase-9. These apparently conflicting data for the dATP/ATP requirement are probably explained by the pre-formation of Apaf-1M368L oligomeric complex within the cell after exposure to endogenous dATP/ATP.

ATP hydrolysis is only required for a brief period of time for Apaf-1 to activate procaspase-9

Next we performed experiments to determine whether dATP/ATP hydrolysis is continuously required for Apaf-1-mediated activation of procaspase-9. To do this, we added the non-hydrolyzable ATP analog, ATP- γ S, to the procaspase-9 activation assay at various times, incubating the reaction for a total of 30 min. As depicted in Figure 5, procaspase-9 activation was greatly inhibited by ATP- γ S when added at the beginning of the reaction. ATP- γ S inhibited procaspase-9 activation by ~70% when added after 10 s and by ~30% after 20 s (Figure 5). ATP- γ S minimally inhibited or could no longer inhibit procaspase-9 activation when it was added after 40 s (Figures 5). These experiments suggest that dATP/ATP hydrolysis is required for a brief period of time to activate procaspase-9.

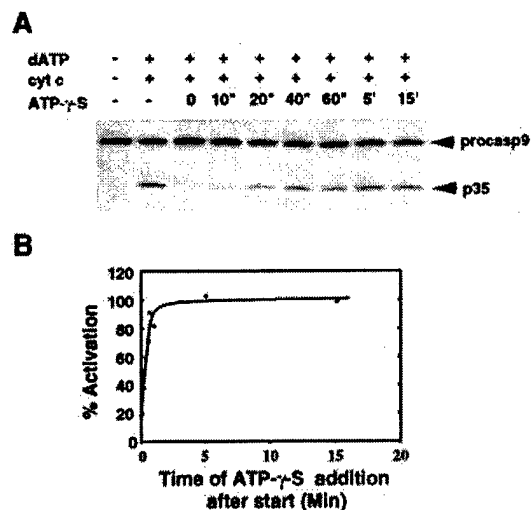


Fig. 5. dATP hydrolysis is only required for a short time in Apaf-1-mediated procaspase-9 activation. Two micrograms of Apaf-1-containing extract was mixed with 1 μ l [35 S]methionine-labeled procaspase-9 on ice in the presence of 0.1 mM dATP and 0.2 μ g of cytochrome *c*. One millimolar ATP- γ S was added to the reaction mixture at different time intervals after the tubes containing reaction mixtures were transferred into 30°C waterbath. The reaction was incubated for a total of 30 min at 30°C. (A) Procaspase-9 activation profile in the presence of ATP- γ S when added at different times after the beginning of the reactions. (B) Time course of inhibition of procaspase-9 processing by ATP- γ S. The amount of unprocessed and processed caspase-9 (p35) forms were scanned and quantitated using a phosphorimager. First, the amount of p35 processed forms was normalized to full-length procaspase-9 and added to the corresponding unprocessed procaspase-9 to obtain the total amount of procaspase-9 used in each lane. Then, p35 bands were normalized relative to total procaspase-9 in each lane. Finally, normalized p35 fragments were divided by the p35 fragment without ATP- γ S (positive control).

Apaf-1M368L exhibits enhanced ability to induce apoptosis

The experiments shown in Figure 1D indicate that the Apaf-1M368L mutant can activate procaspase-9 independently of cytochrome *c*. To determine the significance of these findings for induction of apoptosis, we transiently transfected wt and mutant Apaf-1 constructs into 293T cells and assessed their ability to induce apoptosis 19 h after transfection. Wt Apaf-1 failed to induce cell death at low concentrations, but induced low-level apoptosis at higher concentrations of plasmid DNA (Figure 6). In contrast, Apaf-1M368L induced significant apoptosis even at low levels of plasmid DNA and exhibited greater proapoptotic activity than wt Apaf-1 at higher levels of plasmid (Figure 6B). The P-loop mutant of Apaf-1 (K160R) deficient in Apaf-1 self-association and procaspase-9 binding failed to induce apoptosis at all concentrations of plasmid tested (Figure 6B). Apaf-1M368L-induced apoptosis was effectively inhibited by a catalytically inactive caspase-9 mutant (C287S) that acts as a dominant-negative inhibitor (Figure 6B) (Li *et al.*, 1997; Pan *et al.*, 1998), indicating that apoptosis induced by Apaf-1M368L is caspase-9 dependent. Significantly, the Apaf-1 double mutant, M368L/K160R, did not induce apoptosis consistent with a requirement of dATP/ATP hydrolysis for Apaf-1M368L to self-associate and binding to procaspase-9 (Figure 6C).

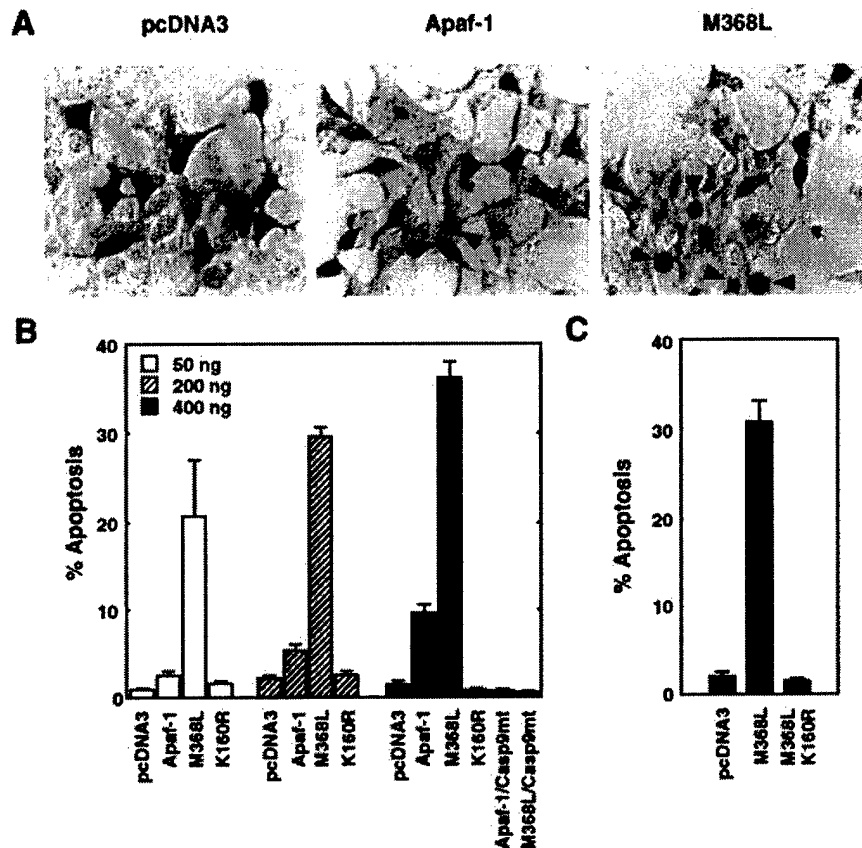


Fig. 6. Induction of apoptosis by wt and Apaf-1 mutants. (A) Representative field of 293T cells transfected with 400 ng of indicated control and Apaf-1 constructs and stained for β -galactosidase activity. Darker cells represent transfected and β -galactosidase-positive cells. The arrowheads indicate round-up apoptotic cells with membrane blebbing. (B) Apaf-1M368L induces apoptosis in 293T cells. 293T cells were transiently transfected with a reporter pcDNA3-galactosidase plus the indicated plasmids. The results represent the percentage of blue cells that exhibit morphological features of apoptosis and are given as the mean \pm SD of triplicate cultures. Casp9mt represents a catalytically inactive mutant of procaspase-9 (C287S). (C) Apaf-1M368L/K160R failed to induce apoptosis. Four-hundred nanograms of indicated plasmids were used to transiently transfect 293T cells as in (B).

Apaf-1(1-570) fails to induce apoptosis and activate procaspase-3

Like the Apaf-1M368L mutant, Apaf-1 mutants lacking the WDR can activate procaspase-9 independent of cytochrome *c* and dATP (Figure 1C) (Hu *et al.*, 1998a,b; Srinivasula *et al.*, 1998). Therefore, we compared the ability of Apaf-1M368L and Apaf-1(1-570) to induce apoptosis. Significantly, Apaf-1(1-570) did not kill 293T cells while Apaf-1M368L induced significant apoptosis in transfected cells (Figure 7A). Immunoblotting analysis with anti-caspase-9 antibody showed that both Apaf-1M368L and Apaf-1(1-570) induced processing of endogenous procaspase-9 into its signature p35 fragment (Figure 7B, upper panel), indicating that the differential activity of these Apaf-1 mutants cannot be explained by lack of procaspase-9 activation. To assess the activation of procaspase-3, a downstream caspase activated by caspase-9, we immunoblotted the same extracts with anti-caspase-3 antibody. Expression of Apaf-1M368L induced processing of procaspase-3, as determined by the appearance of the p18 subunit and reduction of procaspase-3 levels (Figure 7B, lower panel, reduced levels of procaspase-3 could be observed in a shorter exposure; data not shown). Significantly, procaspase-3 from cells transfected with Apaf-1(1-570) remained unprocessed even though procaspase-9 was cleaved (Figure 7B). Thus,

the differential proapoptotic activity exhibited by the constitutively active Apaf-1 mutants, Apaf-1(1-570) and Apaf-1M368L, might be explained by the differential processing of procaspase-3. To gain more insight into this mechanism, we further assessed the activation of procaspase-3 and procaspase-9 in cytosolic extracts prepared from Apaf-1M368L and Apaf-1(1-570)-transfected cells. In Apaf-1M368L extracts, procaspase-9 could be effectively processed into the signature p35 proteolytic fragment resulting from autoactivation of procaspase-9 and p37, a fragment resulting from cleavage of procaspase-9 by active caspase-3 (Figure 7C, upper panel) (Srinivasula *et al.*, 1998). In contrast, procaspase-9 was only cleaved into p35 but not p37 in the Apaf-1(1-570) extract (Figure 7C), providing additional evidence that caspase-3 was not activated in extracts expressing Apaf-1(1-570). To verify this result, we assessed the processing of procaspase-3 in the same extracts. The analysis confirmed that Apaf-1M368L but not Apaf-1(1-570) promoted procaspase-3 activation (Figure 7C, lower panel).

Apaf-1M368L but not Apaf-1(1-570) can recruit procaspase-3 to the Apaf-1-procaspase-9 complex through procaspase-9

Next we wanted to determine the mechanism by which procaspase-3 is activated in extracts from Apaf-1M368L

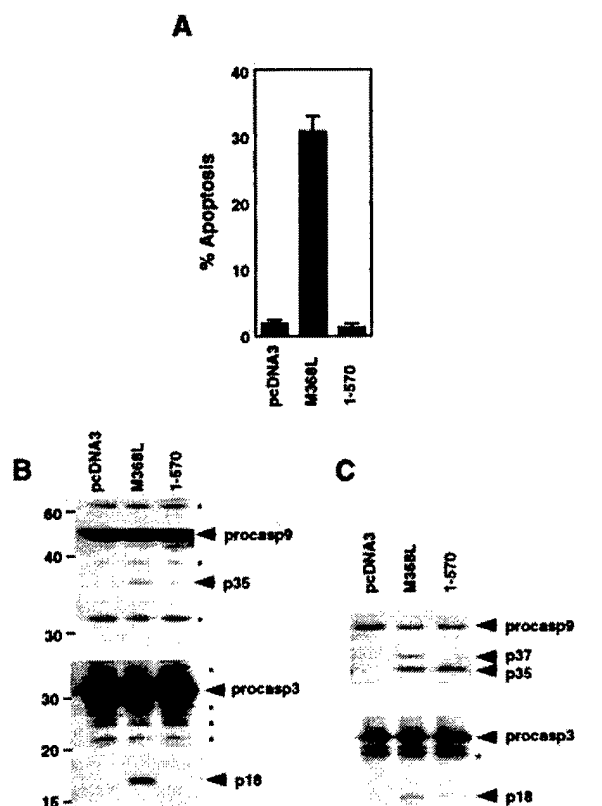


Fig. 7. Apaf-1(1-570) fails to induce apoptosis and activate procaspase-3. (A) Apaf-1(1-570) is unable to induce apoptosis of 293T cells. 293T cells were transiently transfected with a reporter pcDNA3-galactosidase plus 0.4 μ g of the indicated plasmids. The results represent the percentage of blue cells that exhibit morphological features of apoptosis and are given as the mean \pm SD of triplicate cultures. (B) Procaspase-9 but not procaspase-3 is processed in Apaf-1(1-570)-transfected 293T cells. Total cell lysates from 293T cells as indicated in (A) were separated on SDS-PAGE gel and immunoblotted for caspase-9 (upper panel) and caspase-3 (lower panel). Molecular weight markers are indicated on the left of each blot. (C) Both Apaf-1M368L and Apaf-1(1-570) can activate procaspase-9 but only Apaf-1M368L is able to activate procaspase-3. Forty micrograms of cytosolic extracts containing Apaf-1M368L or Apaf-1(1-570) were incubated with [35 S]methionine-labeled procaspase-9 (upper panel) or procaspase-3 (lower panel) in the absence of cytochrome c and dATP at 30°C for 30 min. Arrows indicate immature and processed forms of caspase-9 (upper panel) and caspase-3 (lower panel). p35 and p18 represent processed forms of procaspase-9 and -3 respectively. Asterisks indicate non-specific bands.

transfected cells but not from those expressing Apaf-1(1-570). One possibility we considered is that procaspase-3 could be recruited to the Apaf-1M368L–procaspase-9 complex but not to the Apaf-1(1-570)–procaspase-9 complex. To examine this, we immobilized various Apaf-1 mutants on agarose beads in the presence or absence of procaspase-9 and incubated the immobilized Apaf-1 or Apaf-1–procaspase-9 complexes with *in vitro* translated procaspase-3. In the absence of procaspase-9 (C2827S), procaspase-3 interacted weakly with Apaf-1M368L, while procaspase-3 did not associate with Apaf-1(1-570) or Apaf-1(479-1248) (Figure 8A). The presence of procaspase-9 greatly enhanced the binding of procaspase-3 to Apaf-1M368L (Figure 8A). In contrast, procaspase-3 did not bind to Apaf-1(1-570) even in the presence of procaspase-9 (Figure 8A). To determine whether procaspase-3 interacts with procaspase-9 alone

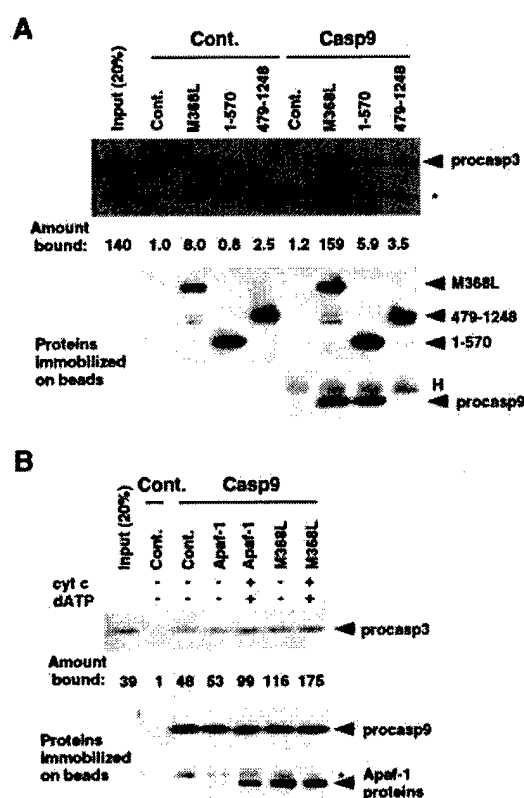


Fig. 8. Procaspase-3 is recruited into the Apaf-1–caspase-9 complex but not into the Apaf-1(1-570)–caspase-9 complex. (A) Procaspase-3 is recruited into Apaf-1M368L–caspase-9 complex. Apaf-1 proteins were immobilized on agarose beads and packed into gel filtration columns as described in Materials and methods. *In vitro*-translated [35 S]methionine-labeled procaspase-3 was loaded onto the beads. After extensive washing proteins bound to the beads were eluted, separated by SDS-PAGE and transferred to nitrocellulose membrane. (B) Procaspase-3 binds to procaspase-9 directly. Experiments were performed as in (A) except procaspase-9 (C2827S) was immobilized on agarose beads. Procaspase-3 bound was detected by autoradiography and Apaf-1 proteins and procaspase-9 (C2827S) were detected by immunoblotting. The upper panels show an autoradiograph of procaspase-3 bound and amounts of bound procaspase-3 as quantitated with a phosphorimager (procaspase-3 bound to control beads was arbitrarily designated as 1). Apaf-1 proteins and procaspase-9 (C2827S) bound to beads were shown in lower two panels. Asterisks indicate non-specific bands. H, immunoglobulin heavy chain; Cont., control lysates transfected with pcDNA3.

or with the Apaf-1M368L–procaspase-9 complex, we performed similar experiments with immobilized procaspase-9 (Figure 8B). As shown in Figure 8B, procaspase-3 did bind to procaspase-9 in the absence of Apaf-1. Addition of Apaf-1M368L or wt Apaf-1 in the presence of cytochrome c and dATP enhanced the binding of procaspase-3 to immobilized procaspase-9 by 2- to 4-fold (Figure 8B). This effect is presumably due to increased recruitment of procaspase-9 to the complex by binding to Apaf-1M368L or Apaf-1 in the presence of cytochrome c and dATP. A direct interaction between procaspase-3 and procaspase-9 was also confirmed by co-immunoprecipitation analysis (data not shown). Thus, wt Apaf-1 and Apaf-1M368L but not Apaf-1(1-570) can recruit procaspase-3 to the Apaf-1–procaspase-9 complex through procaspase-9.

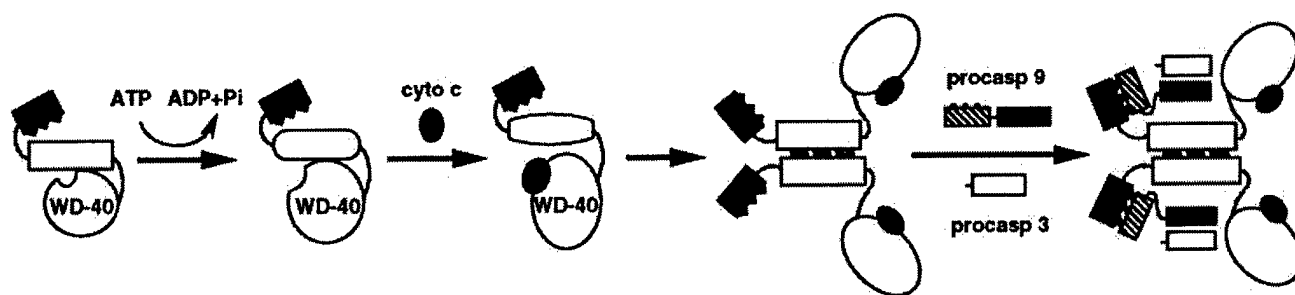


Fig. 9. A model depicting the mechanism of apoptosome formation and procaspase-9 activation. Apaf-1 undergoes sequential conformational changes induced by ATP hydrolysis and cytochrome *c* binding and acquires the ability to oligomerize. Subsequent binding of procaspase-9 to the Apaf-1 oligomer results in recruitment of procaspase-3 and possibly procaspase-7 to this complex to form a functional apoptosome. Apaf-1M368L may bypass the first two steps and adopts a conformation that is competent in oligomerizing and binding to procaspase-9 in the presence of dATP. Alternatively, Apaf-1M368L may assume a conformation that bypasses cytochrome *c* binding but still requires a single dATP/ATP hydrolysis event for oligomerization and procaspase-9 binding. This model is presented in greater detail in the Discussion.

Discussion

In this report we used Apaf-1 and various mutants to elucidate the mechanism by which Apaf-1 mediates procaspase-9 activation and induces apoptosis. Our studies revealed that the binding of cytochrome *c* to Apaf-1 requires dATP/ATP hydrolysis. We also demonstrated that two essential steps involved in procaspase-9 activation, Apaf-1 self-association and procaspase-9 recruitment to Apaf-1, require dATP/ATP hydrolysis and cytochrome *c* binding to Apaf-1. These steps are important for proapoptotic activity since Apaf-1 mutants deficient in dATP/ATP hydrolysis or cytochrome *c* binding are impaired in their ability to promote procaspase-9 activation and apoptosis. Finally, we provide evidence that procaspase-3 is recruited into the Apaf-1–procaspase-9 complex via interaction with procaspase-9. The WDR may play an important role in the recruitment of procaspase-3 to the Apaf-1–procaspase-9 complex, since mutant Apaf-1(1–570) lacking the WDR can bind to procaspase-9 but cannot recruit procaspase-3 and is deficient in apoptosis.

Our observations that the WDR with an extra WD-40 repeat can interact with Apaf-1(1–570) and inhibit activation of procaspase-9 mediated by Apaf-1(1–570) (Figure 1B and C) support conclusions of our previous paper obtained with the original Apaf-1 cDNA clone (Hu *et al.*, 1998b). Based on the results of our present study and previous studies (Hu *et al.*, 1998b; Srinivasula *et al.*, 1998), we provide a model depicting the events that lead to the formation of a functional apoptosome (Figure 9). In this model, dATP/ATP hydrolysis induces a conformational change so that Apaf-1 acquires the ability to bind cytochrome *c*. Upon cytochrome *c* binding, Apaf-1 undergoes further conformational changes to allow Apaf-1 oligomerization and CARD exposure. Procaspase-9 is then recruited into Apaf-1 oligomers via CARD–CARD homophilic interactions, and procaspase-3 is recruited into the complex via interaction with procaspase-9. Once recruited to the apoptosome, procaspase-9 is activated via autocatalysis by Apaf-1 oligomerization-induced proximity of multiple procaspase-9 molecules (Hu *et al.*, 1998b; Srinivasula *et al.*, 1998). Procaspase-9 might be cleaved and activated by adjacent procaspase-9 molecules (intermolecular catalysis). Alternatively, procaspase-9, forming part of the apoptosome, might adopt a conformation that results in intramolecular

cleavage. Activated caspase-9 then cleaves and activates procaspase-3 recruited to the apoptosome. Once the functional apoptosome is formed, dATP/ATP hydrolysis would no longer be required for procaspase-9 recruitment, activation or release of active caspase-9, as suggested by our finding that dATP/ATP hydrolysis is only required for a brief period of time in order for Apaf-1 to activate procaspase-9. A model of Apaf-1 function that is consistent with our findings has been proposed recently based on studies with purified Apaf-1 and procaspase-9 proteins (Zou *et al.*, 1999).

In this study, we generated and characterized an Apaf-1 mutant that activates procaspase-9 independent of cytochrome *c*. Apaf-1M368L constitutively self-associates when synthesized in living cells. However, additional mutation of Lys160 to Arg abrogated its ability to self-associate and bind procaspase-9, as well as its capacity to promote procaspase-9 activation and apoptosis. Although the precise mechanism that accounts for its constitutional activation remains to be determined, these results suggest that Apaf-1M368L may bind and hydrolyze dATP/ATP *in vivo*, and induce Apaf-1 oligomerization and procaspase-binding and activation as intracellular dATP or ATP levels are available in cells at concentrations of 10 μ M and 10 mM, respectively (Skoog and Bjursell, 1974). The ability of Apaf-1 to bind and hydrolyze dATP/ATP was demonstrated by a recent paper that was published while this paper was under review (Zou *et al.*, 1999). In the absence of dATP/ATP hydrolysis, the ability of wt Apaf-1 to oligomerize and to bind procaspase-9 might be repressed by an interaction between its N-terminal region and the WDR (Figure 1B and C) (Hu *et al.*, 1998b). One role of dATP/ATP hydrolysis might be to induce a conformational change that enables cytochrome *c* to bind Apaf-1. This hypothesis is supported by our finding that cytochrome *c* binding to Apaf-1 requires dATP/ATP hydrolysis. Binding of cytochrome *c* to the WDR may disrupt or alter the negative interaction between the N-terminal region and the WDR, allowing Apaf-1 to oligomerize and to bind procaspase-9. This model is supported by the analysis of the Apaf-1M368L mutant. This mutant exhibited reduced interaction between the N-terminal region and the WDR, bypassing the requirement of cytochrome *c* to disrupt the negative interaction. The lack of requirement for cytochrome *c* suggests that Apaf-

1M368L spontaneously adopts a conformation that is competent for oligomerization and procaspase-9 binding in the presence of dATP/ATP. Mutation of Met368 to Leu, therefore, might mimic conformational changes induced by dATP hydrolysis and cytochrome *c* binding. Thus, our findings that Apaf-1M368L still requires dATP/ATP binding or hydrolysis suggest that a second dATP/ATP binding or hydrolysis event may be required. Alternatively, Apaf-1M368L may assume a conformation that bypasses cytochrome *c* binding but still requires a single dATP/ATP hydrolysis event for oligomerization and procaspase-9 binding. CED-4, the *C.elegans* homologue of Apaf-1, lacks the WDR implying that CED-4 could constitutively activate CED-3, unless repressed by its physical association with CED-9. This model is supported by genetic analysis of *C.elegans* that showed that loss-of-function mutants of *ced-9* are lethal due to increased developmental cell deaths, but can be rescued by loss-of-function mutations in *ced-4* (Hengartner *et al.*, 1992). Furthermore, biochemical experiments in mammalian cells have revealed that the ability of CED-4 to activate CED-3 is inhibited by the association of CED-9 with CED-4 (Seshagiri and Miller, 1997; Wu *et al.*, 1997b). Interestingly, the equivalent position of Met368 is conserved in mouse Apaf-1 but is a leucine residue in CED-4. These observations suggest that cytochrome *c* is probably not a factor required for CED-4-mediated CED-3 activation. However, additional studies to test this hypothesis need to be performed in *C.elegans*.

Apaf-1M368L acts as a gain-of-function mutation in that, unlike wt Apaf-1, it activates procaspase-9 independent of cytochrome *c* and dATP *in vitro* and exhibits enhanced ability to induce apoptosis *in vivo*. In contrast, another mutant, Apaf-1(1-570), activates procaspase-9 independent of cytochrome *c* and dATP *in vitro*, but fails to induce apoptosis. Our results indicate that procaspase-9 complexed with Apaf-1M368L, but not procaspase-9 complexed with Apaf-1(1-570), can recruit and activate procaspase-3, providing an explanation for the differential phenotypes. Our studies support a model whereby procaspase-3 is recruited into the apoptosome formed by the Apaf-1-procaspase-9 complex via its interaction with procaspase-9. Apaf-1(1-570) lacking the WDR binds and activates procaspase-9 suggesting that its inability to recruit procaspase-3 is independent of these activities. Several possibilities could be envisioned to explain these results. First, conformational changes in the procaspase-9 bound to wt Apaf-1 and Apaf-1M368L could be critical for the recruitment of procaspase-3. Procaspase-9 bound to Apaf-1(1-570) might assume a conformation that is unsuitable for procaspase-3 recruitment. Another possibility is that the WDR, which is lacking in Apaf-1(1-570), might be involved in the recruitment of procaspase-3 to the Apaf-1-procaspase-9 complex. In the absence of the WDR, Apaf-1(1-570) may be unable to induce the conformational changes of procaspase-9 required for recruitment of procaspase-3.

The cytochrome *c*/Apaf-1/caspase-9/caspase-3 pathway is now known to contribute to apoptosis induced by various stresses, including UV and γ -irradiation and chemotherapeutic drugs (Hakem *et al.*, 1998; Yoshida *et al.*, 1998). In addition, inactivation of either Apaf-1 or caspase-9 in this pathway contributes to cellular trans-

formation and tumorigenesis (Soengas *et al.*, 1999). Under normal conditions, cytochrome *c* is unavailable to induce procaspase-9 activation since it resides in the intermembrane space of the mitochondria. After apoptotic stimuli lead to mitochondrial damage, cytochrome *c* is released and can activate procaspase-9 in an Apaf-1-dependent manner (Zou *et al.*, 1997; Fearnhead *et al.*, 1998). Prosurvival Bcl-2 family members are overexpressed in many tumors and inhibit caspase-9-mediated apoptosis by binding and inhibiting procaspase-9 activation directly and/or preventing the release of cytochrome *c* from mitochondria (Kharbanda *et al.*, 1997; Kim *et al.*, 1997; Kluck *et al.*, 1997; Yang *et al.*, 1997; Hu *et al.*, 1998a). Therefore, it will be interesting to examine whether prosurvival Bcl-2 family members like Bcl-X_L can directly inhibit Apaf-1M368L-mediated procaspase-9 activation. If Bcl-X_L can no longer exert its inhibitory effect on Apaf-1M368L-mediated activation of procaspase-9, the ability of Apaf-1M368L to activate procaspase-9 in the absence of cytochrome *c* could provide a potential method of inducing apoptosis in cancer cells in which cytochrome *c* release is blocked by overexpression of anti-apoptotic members of the Bcl-2 family.

Materials and methods

Plasmids and reagents

The Apaf-1XL cDNA was isolated by RT-PCR amplification from RNA template isolated from 293T cells and cloned into the pcDNA3 plasmid. The nucleotide sequence of the Apaf-1XL cDNA has been submitted to the DDBJ/EMBL/GenBank database (accession No. AF149794). pcDNA3-Apaf-1XL-M368L and pcDNA3-Apaf-1XL-K160R were generated by replacing the *Bst*EII-*Eco*RV fragment of pcDNA3-Apaf-1XL with the corresponding fragments from pcDNA3-Apaf-1(1-559M357L) and pcDNA3-Apaf-1(1-559K149R), respectively (Hu *et al.*, 1998b). pcDNA3-Apaf-1XL-M368L/K160R was constructed by ligation of a *Bst*EII-*Xba*I fragment from pcDNA3-Apaf-1(1-559K149R) and a *Xba*I-*Eco*RV fragment from pcDNA3-Apaf-1(1-559M357L) into *Bst*EII-*Eco*RV-digested pcDNA3-Apaf-1XL. All the constructs were verified by nucleotide sequencing. Polyclonal anti-Apaf-1 antibody was kindly provided by Xiaodong Wang, Southwestern Medical School, Dallas, TX. Polyclonal anti-caspase-9 and anti-caspase-3 antibodies were gifts from Donald Nicholson (Merck).

Transfection, immunoprecipitation and Western blot analysis

Human embryonic kidney 293T cells (2-53 10^6) were transfected with 5 μ g of the indicated plasmid DNA by the calcium phosphate method as reported (Inohara *et al.*, 1997). Cytosolic extracts were made essentially as described previously (Hu *et al.*, 1998a). Protein immunoprecipitation and Western blot analysis with relevant antibodies were performed as described previously (Inohara *et al.*, 1997). The proteins were detected by an enhanced chemiluminescence system (ECL; Amersham).

In vitro caspase-9 and caspase-3 assay

Cytosolic extracts were prepared and stored frozen at -80°C . Procaspase-9 was translated *in vitro* from a pcDNA3-caspase-9 plasmid in the presence of [^{35}S]methionine (Amersham) with a Promega TNT transcription/translation kit and purified through a desalting column free of radioactive methionine and ATP (Pharmacia). Procaspase-3 was translated *in vitro* using the same kit. To avoid contaminating dATP/ATP from cytosolic extracts in testing cytochrome *c* and dATP-dependent activation of procaspase-9 by Apaf-1 proteins, the cytosolic extracts were passed through a desalting column (Pharmacia). We incubated 2 μ g (Figures 1D and 5A) or 40 μ g (Figure 7C) of cellular extracts with or without dATP (1 mM) or bovine cytochrome *c* (0.2 μ g, Sigma) in the presence of 1 μ l *in vitro* translated procaspase-9 in a final volume of 25 μ l. The mixtures were incubated for 30 min at 30°C and the reactions stopped by adding 53 SDS loading buffer and boiling for 5 min.

Apoptosis assay

293T cells (13×10^5) were seeded in each well of 12-well plates. After 18 h, cells were transiently transfected with 0.2 µg of the reporter pcDNA3-galactosidase plasmid plus indicated amount of test plasmids, as reported (Hu *et al.*, 1998a). pcDNA3 was used to adjust total plasmid DNA to an equal amount. The percentage of apoptotic cells was determined 19 h after transfection in triplicate cultures as described previously (Hu *et al.*, 1998a).

Procaspase-3 recruitment assay

Cytosolic extracts containing Myc-tagged Apaf-1 proteins and control or caspase-9 extracts were incubated with either anti-c-Myc (to immobilize Apaf-1 protein) or anti-HA (to immobilize procaspase-9) antibodies and protein A/G-agarose beads (1:1) for 2 h. The beads were washed three times with 1 ml NP-40 buffer (Inohara *et al.*, 1997) and once with 1 ml buffer A (Hu *et al.*, 1998a). Apaf-1- or procaspase-9-coated beads were packed into disposable gel filtration columns. Then *in vitro* translated caspase-3 was loaded onto the beads and washed four times with 1 ml buffer A. Bound proteins were eluted with 50 µl of hot 13 SDS loading buffer. We quantitated bound caspase-3 using a PhosphorImager from Molecular Dynamics.

Acknowledgements

We would like to thank Xiaodong Wang, Donald Nicholson, Nancy Thornberry and Margarita Garcia-Calvo for generous gifts of reagents. We thank Naohiro Inohara and Luis del Peso for thoughtful discussions and critical review of the manuscript. This work was supported by grant CA-64556 from the National Institutes of Health and by grant DAMD17-96-609 from US Army Medical Research Command. Y.H. was supported by the National Institutes of Health Postdoctoral Training Grant 2T32HL07517. M.A.B. was supported by a predoctoral fellowship from the US Army Medical Research Command. G.N. is the recipient of Research Career Development Award CA-64421 from the National Institutes of Health.

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